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이학박사학위논문

**Embryonic Neurogenesis 에서  
스트레스 관련 인자들의 역할  
Roles of Stress-related Factors  
in Embryonic Neurogenesis**

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## ABSTRACT

Stress exposure during development can lead to severe neurological diseases in infants and children. Many genes are known to be related to stress induction, but their roles in brain development have not been clearly elucidated. To rapidly screen for biologically meaningful factors involved in brain development, I first selected several genes that are known to be up-regulated in the fetal brain during prevalent stressed conditions such as prenatal infection and alcohol exposure, including nuclear factor (erythroid-derived 2)-like 2 (Nrf2), glycogen synthase kinase 3 (GSK3)  $\alpha$ , GSK3 $\beta$ , interferon-gamma (IFN- $\gamma$ ), and sterol-C4-methyl oxidase-like gene (SC4MOL). These genes were overexpressed in primary embryonic day (E) 14.5 murine neural progenitor cells (NPCs) using retroviral vectors. The percentage of neuronal cells was measured by TuJ1 expression. Using this method, GSK3 $\beta$  and IFN- $\gamma$  were found to have strong negative effects on neurogenesis, and they were further characterized *in vitro* and *in vivo* using various molecular techniques.

GSK3 is known as an important regulator during the proliferation and differentiation of NPCs, but the roles of the isoforms of this molecule (GSK3 $\alpha$  and GSK3 $\beta$ ) have not been clearly defined. Thus, the functions of GSK3 $\alpha$  and GSK3 $\beta$  in the context of neuronal differentiation of NPCs were characterized. Treatment of primary NPCs with a GSK3 inhibitor (SB216763) resulted in an increase in the percentage of TuJ1-positive immature neurons, suggesting an inhibitory role of GSK3 in embryonic neurogenesis. Downregulation of GSK3 $\beta$  expression increased the percentage of TuJ1-positive cells, whereas the knock-down of GSK3 $\alpha$  appeared to have no effect. Mutant GSK3 $\beta$  (Y216F) failed to suppress neuronal differentiation, indicating that the kinase activity of GSK3 $\beta$  is important for this regulatory function. Similar results were obtained *in vivo* when a retroviral vector expressing GSK3 $\beta$  was delivered to E9.5

mouse brains. In addition, SB216763 was found to block the rapamycin-mediated inhibition of neuronal differentiation of NPCs. Taken together, these data demonstrate that GSK3 $\beta$ , but not GSK3 $\alpha$ , negatively controls the neuronal differentiation of NPCs and that GSK3 $\beta$  may act downstream of the mTORC1 signaling pathway.

IFN- $\gamma$  is one of the critical cytokines released by host immune cells upon infection. Despite the important role(s) of IFN- $\gamma$  in host immune responses, there have been no *in vivo* studies of the effects of IFN- $\gamma$  on brain development, and the results obtained from many *in vitro* studies have been controversial. Treatment of E14.5 murine NPCs with IFN- $\gamma$  resulted in a decrease in the percentage of TuJ1-positive immature neurons but an increase in the percentage of Nestin-positive NPCs. Similar results were obtained *in vivo*. Treatment of NPCs with a JAK inhibitor or the knock-down of STAT1 expression abrogated the IFN- $\gamma$ -mediated inhibition of neurogenesis. Interestingly, the expression of one of proneural genes, *Neurogenin2* (*Neurog2*) was inhibited dramatically upon IFN- $\gamma$  treatment, and cells overexpressing *Neurog2* did not respond to IFN- $\gamma$ . Both IFN- $\gamma$  treatment and overexpression of the constitutively active form of STAT1 reduced the *Neurog2* promoter activity by nearly half. These results suggest that IFN- $\gamma$  inhibits the neuronal differentiation of NPCs by negatively regulating the expression of *Neurog2* partially at the promoter level via the JAK/STAT1 pathway.

In this thesis work, I identified GSK3 $\beta$  and IFN- $\gamma$  as negative controllers of neuronal differentiation, which act downstream of the mTORC1 signaling pathway and upstream of the JAK/STAT1 pathway, respectively. This is the first study to clearly distinguish the roles of GSK3 isoforms in the context of neuronal differentiation and to investigate the effects of IFN- $\gamma$  on embryonic neurogenesis *in vivo*. The findings from this thesis may provide insights into the mechanism of action of different stress-related factors during the early period of brain development, especially the initiation of



neurogenesis and the possible consequences of congenital stress exposure.

**Keywords:** Embryonic Neurogenesis, NPC differentiation, Prenatal Stress, Nrf2, GSK3, IFN- $\gamma$ , SC4MOL

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## ABBREVIATIONS

APC	Adenomatous polyposis coli
APP	Amyloid precursor protein
ARE	Antioxidant response element
AS-C	Achaete-scute complex
Ato	Atonal
CBF1	C-promoter binding factor 1
CK1 $\alpha$	Casein kinase 1 $\alpha$
CNC-bZip	Cap 'n' collar type of basic region leucine zipper factor
DMEM	Dulbecco's modified Eagle's medium
Dsh	Dishevelled
E	Embryonic day
eGFP	Enhanced GFP
FASDs	Fetal alcohol spectrum disorders
Fz	Frizzled
GAS	IFN- $\gamma$ activating sequence
GFAP	Glial fibrillary acid protein
GOI	Gene of interest
GSK3	Glycogen synthase kinase
H&E	Hematoxylin and eosin
Hes	Hairy and enhancer of split paralogues
HO-1	Heme oxygenase-1
Id	Inhibitor of differentiation
IF	Immunofluorescence
IFN- $\gamma$	Interferon-gamma
IHC	Immunohistochemistry
INPs	Intermediate neurogenic progenitor cells



KO	Knock-out
LRP	Lipoprotein receptor-related protein
MAPKAP-K1	MAPK-activated protein kinase 1
Mash	Mammalian achaete-scute homologue
MIA	Maternal immune activation
MSCV	Murine stem cell virus
mTORC1	Mammalian target of rapamycin complex 1
Neurog2	<i>Neurogenin 2</i>
Neurogs	Neurogenins
NICD	Notch intracellular domain
NPCs	Neural progenitor cells
Nqo1	NAD(P)H quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid derived 2-related factor 2
Olig	Oligodendrocyte lineage transcription factor
Pax6	Paired box 6
PBS	Phosphate buffered saline
PC	Positive control
PKB	Protein kinase B
PP	Protein phosphatase
Ptc	Patched 1 receptor
ROR	Receptor tyrosine kinase-like orphan receptor
ROS	Reactive oxygen species
RT	Room temperature
Ryk	Receptor-like tyrosine kinase
SC4MOL	Sterol-C4-methyl oxidase-like gene
SD	Standard deviation
Shh	Sonic hedgehog
Smo	Smoothened

STAT1ca	Constitutively active form of STAT1
SVZ	Subventricular zone
TCF/LEF	T-cell factor/lymphoid enhancing factor
TSC	Tuberous sclerosis complex
UIGD	Ultrasound image-guided gene delivery
UTR	Untranslated region
VZ	Ventricular zone

# **CHAPTER I**

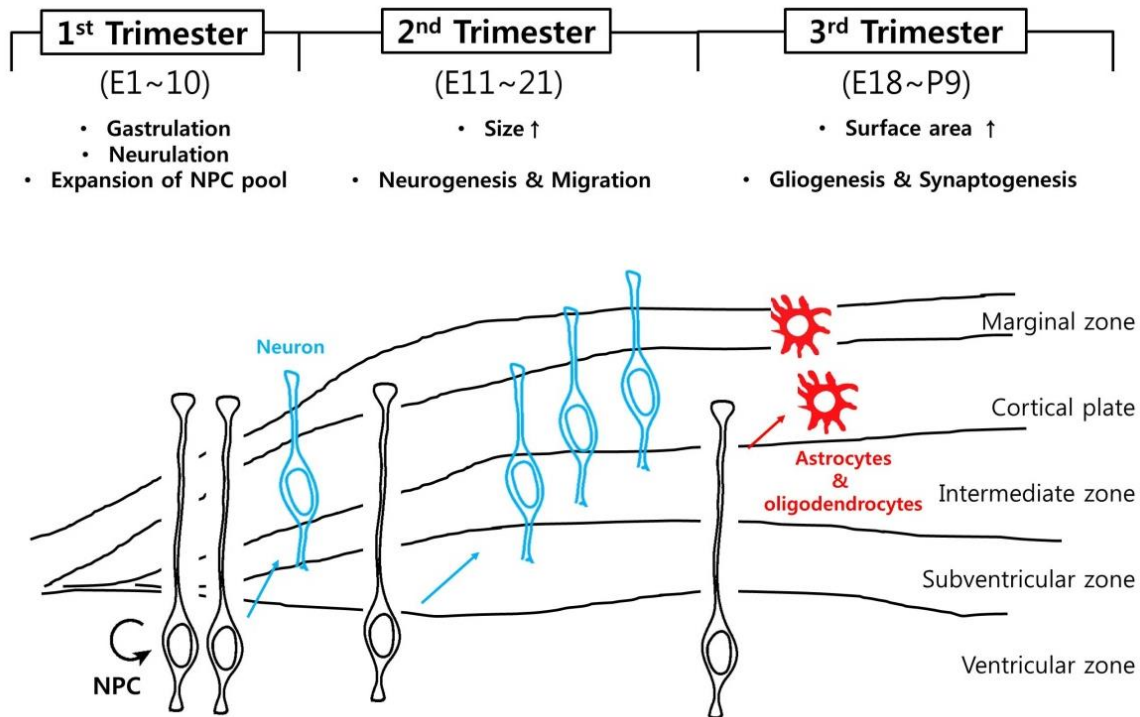
## **Introduction**

During brain development, various cellular pathways regulate numerous developmental processes including the proliferation, differentiation, and migration of cells, which occur concurrently in a spatiotemporal manner. Radical dynamic changes occur during a relatively short period of time; therefore, slight variations in genetic or environmental factors can cause severe developmental abnormalities. The degree and type of many congenital disorders vary greatly, and the underlying causes are unknown in more than 50% of the cases [1]. The main causes are believed to be environmental factors, i.e., stresses to which the mother or fetus have been exposed to such as smoking, radiation, lack of nutrients, and medication misuse. Among the many environmental causes, the two most prevalent stress conditions in our daily lives are prenatal alcohol exposure and congenital infection, which are associated with the increased risk of many neurological defects in newborn children such as microcephaly, delayed development, mental retardation, schizophrenia, and autism [2-5]. The identification of the biological processes responsible for these defects is critical yet difficult because a complete scientific understanding of embryonic brain development is lacking. As the first step to understand the relationship between environmental stress and neurodevelopment, I screened several stress-related genes to determine their involvement in neuronal differentiation. Factors with clear effects were selected, and their characteristics and roles in the differentiation of neural progenitor cells (NPCs) were further investigated *in vivo* as well as *in vitro*.

## **1. Brain development**

### **1.1 Overview of brain development**

Upon fusion of the sperm and egg, a zygote is formed, and it develops into a single entity through multiple steps over the course of 9 months. This developmental process can be divided into three 3-month-long trimesters (Fig. I-1). The brain starts to form in the 1<sup>st</sup> trimester and continues to develop even after birth. During the 1<sup>st</sup> trimester, the main structures of the brain appear and different parts begin to take shape. The brain grows continually during the 2<sup>nd</sup> trimester, and the major and



**Figure I-1. Overview of brain development.** During the 1<sup>st</sup> trimester, the main structures of the brain start to take shape through gastrulation and neurulation. NPCs undergo symmetric division to increase the size of the NPC pool at the VZ. Between the end of the 1<sup>st</sup> trimester and the beginning of the 2<sup>nd</sup> trimester, neuronal differentiation of NPCs is initiated, and the newly formed neurons start to migrate toward the cortical plate. In the 3<sup>rd</sup> trimester, gliogenesis and synaptogenesis start to occur. During this period, the size and surface area of the brain increase dramatically.

minor grooves are then formed in the 3<sup>rd</sup> trimester, increasing the surface area of the brain [6]. During this period, dramatic morphological changes as well as numerous cellular processes occur [7].

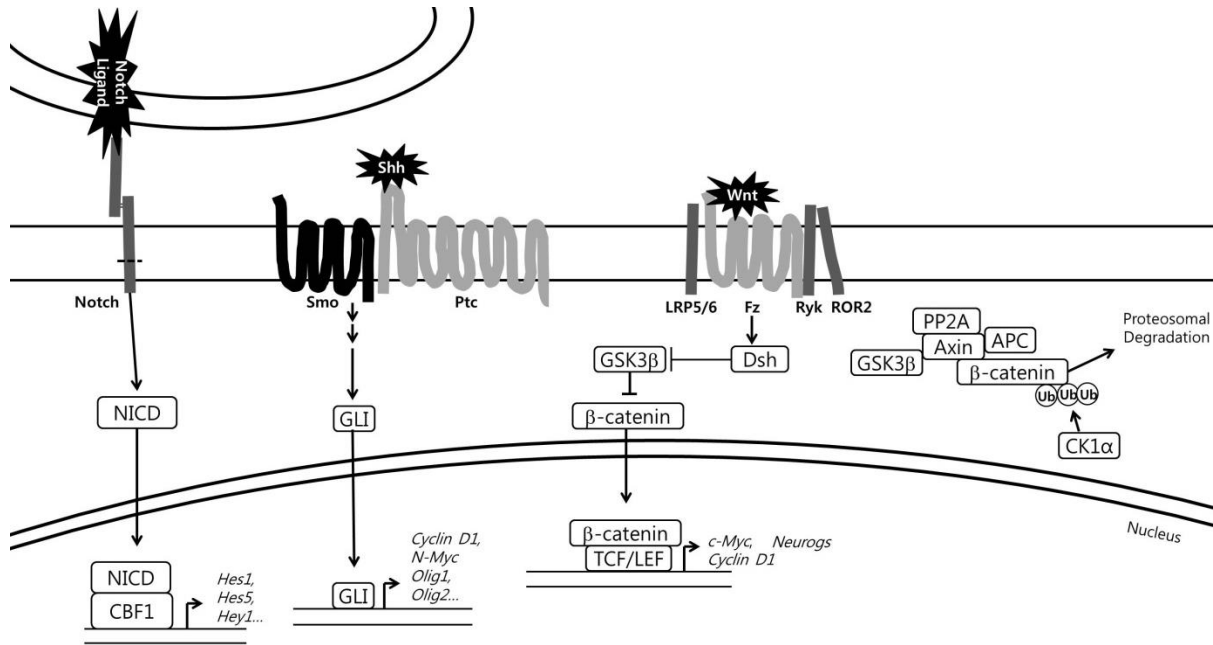
During the 1<sup>st</sup> trimester, NPCs, which can differentiate into different types of neural cells (neurons, astrocytes, and oligodendrocytes), actively proliferate in the ventricular zone (VZ) and increase the size of the NPC pool. In mice, NPCs start to differentiate into neurons at around embryonic day (E) 9. Differentiated neurons then begin to migrate from the VZ towards the cortical plate [8, 9]. In the 3<sup>rd</sup> trimester, synaptogenesis (the formation of synapses between neurons) begins and astrocytes and oligodendrocytes also appear around this time [10, 11]. In mice, the 3<sup>rd</sup> trimester corresponds to the postnatal period; therefore, this process takes place after birth. Brain development and NPC differentiation are executed through the activation, deactivation, and interaction of multiple signaling pathways [12, 13]. The transition to the next step requires various factors; however, the exact mechanism for each factor has not been clearly identified.

## **1.2 Major signaling pathways in NPCs**

A few crucial signaling pathways with major roles in the differentiation of NPCs are illustrated in Fig. I-2 and described in the following.

### **Notch**

Notch is a 300-kDa transmembrane receptor, which exists as a heterodimer and responds to Notch ligand proteins including Delta 1, 3, and 4, or Jagged 1 and 2. The role of Notch in brain development has been studied extensively, and factors that play roles in the Notch signaling pathway has been found to be highly expressed in NPCs [14, 15]. After the Notch ligand interacts with the extracellular domain of Notch, gamma-secretase cleaves the intracellular domain of Notch, releasing the Notch intracellular domain (NICD). NICD then translocates to the nucleus and interacts with the DNA binding protein C-promoter binding factor 1 (CBF1), also known as RBPJ or CSL, which allows the transcription of target genes [16]. Notch enhances the proliferation of NPCs and the differentiation



**Figure I-2. Major signaling pathways in NPCs.** Schematic diagrams of the Notch, Shh, and Wnt signaling pathways are shown. When Notch signaling is activated, NICD is released and translocates to the nucleus. NICD then associates with CBF1 and activates its downstream target genes. In the Shh pathway, activated Smo releases Gli, which initiates the transcription of downstream target genes. When the Wnt pathway is activated, Fz activates Dsh, which blocks the proteosomal degradation of β-catenin. β-catenin then associates with TCF/LEF and starts the transcription of its downstream target genes. NICD, Notch intracellular domain; CBF1, C-promoter binding factor 1; Smo, Smoothened; Ptc, Patched 1 receptor; Ci, cubitus interruptus; CtBP LRP5/6, lipoprotein receptor-related protein 5/6; Fz, Frizzled; Ryk, receptor-like tyrosine kinase; ROR2, receptor tyrosine kinase-like orphan receptor 2; Dsh, disheveled; TCF/LEF, T-cell factor/lymphoid enhancing factor; PP2A, protein phosphatase 2A; APC, adenomatous polyposis coli; CK1α, casein kinase 1α.

of astrocytes, but it inhibits neuronal differentiation by increasing the expression of genes in the hairy and enhancer of split paralogues (Hes) family, such as *Hes1* and *Hes5* [17, 18].

### **Sonic hedgehog**

Sonic hedgehog (Shh) is a well-known morphogen that belongs to the Hedgehog family and it is an essential factor in numerous developmental processes including segmentation, organogenesis, and cell differentiation. Shh binds to the membrane-bound Patched 1 receptor (Ptc) and releases the inhibitory activity of Ptc on a membrane embedded protein Smoothed (Smo). Activation of Smo then initiates the nuclear translocation of Gli, which acts as a transcription factor. In the context of NPC differentiation, Gli activates the genes that regulate NPC proliferation and oligodendrocyte differentiation such as cyclin D1, *N-Myc*, and oligodendrocyte lineage transcription factor 1 and 2 (*Olig1* and *Olig2*) [19, 20].

### **Wnt**

Wnt is a secreted glycoprotein that binds to the N-terminal extracellular domain of the transmembrane receptor Frizzled (Fz). Fz interacts with other co-receptors (lipoprotein receptor-related protein 5/6, LRP5/6; receptor-like tyrosine kinase, Ryk; and receptor tyrosine kinase-like orphan receptor 2, ROR2), and binding to Wnt activates the cytoplasmic protein dishevelled (Dsh), which transduces the signal to downstream targets. In the canonical Wnt pathway (Wnt/ $\beta$ -catenin pathway),  $\beta$ -catenin plays a major role as a transcription factor.  $\beta$ -catenin normally forms a destruction complex with Axin, adenomatous polyposis coli (APC), protein phosphatase 2A (PP2A), and GSK3. In the absence of Wnt, Casein kinase 1 $\alpha$  (CK1 $\alpha$ ) ubiquitinates  $\beta$ -catenin and leads to its proteosomal degradation. However, when Wnt signaling is activated, activated Dsh inhibits GSK3 in the destruction complex, allowing the release of  $\beta$ -catenin into the cytoplasm [21]. The accumulated  $\beta$ -catenin proteins translocate to the nucleus and associate with the T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factors, which regulate downstream target genes. Wnt plays various

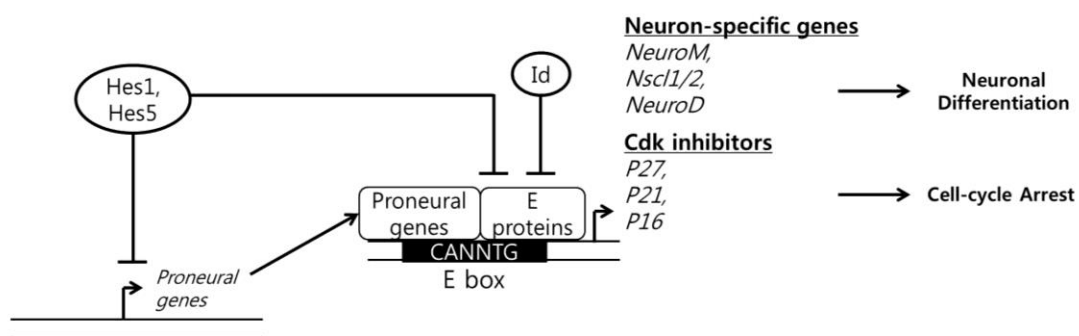


roles in development including body axis formation, cell proliferation, and differentiation. In terms of neurogenesis, it induces the proliferation of early NPCs by regulating cell cycle-related genes, such as *c-myc* and *cyclin D1*, and in the later stage of neurogenesis (E13.5), it induces proneural genes such as *neurogenins* (*Neurogs*) [22-31].

### 1.3 Proneural genes

Downstream target genes of the aforementioned signaling pathways determine the differentiation of NPCs by regulating the expression of proneural genes, which are essential for normal neural differentiation [32, 33]. Proneural genes encode proteins with a characteristic molecular structure, which comprises two alpha helices connected with a short loop. These are called the basic helix-loop-helix (bHLH) transcription factors and most function as transcriptional activators. bHLH factors form homo- or heterodimers with other bHLH factors such as E proteins, and bind to the E-box (CANNTG) consensus sequence (Fig. I-3). The expression of bHLH factors in self-renewing neuroepithelial cells induces commitment to a neuronal fate instead of a glial fate [33]. When these factors are activated, they upregulate the expression of Notch ligands such as *Delta* or *Jagged*, which bind to Notch receptor in neighboring cells, inhibiting their neuronal differentiation.

Proneural genes are divided into two main groups based on their sequence similarity: genes of the achaete-scute complex (AS-C) including the *mammalian achaete-scute homologue 1* and 2 (*Mash1/2*), and the atonal (Ato) group including *Neurog 1*, 2, and *D*, and *Math1* [34]. The deletion of proneural genes delays or impairs neuronal development and causes abnormal cell proliferation or brain morphology. The two main genes that are required and sufficient for the neurogenic program are *Mash1* and *Neurog2*, which are expressed in a non-overlapping pattern. *Mash1* is expressed in the progenitor cells of the ventral telencephalon and olfactory epithelium, whereas *Neurog2* is expressed in the progenitor cells of the dorsal telencephalon and ventral spinal cord. *Mash1* and *Neurog2* are responsible for the generation of different neuronal types (autonomic GABAergic neurons, olfactory receptor neurons, and dopaminergic neurons).



**Figure I-3. Proneural gene pathway.** When proneural genes are expressed, they form homo- or heterodimers with E proteins, and bind to the E-box (CANNTG) consensus sequence. This then activates the expression of neuron-specific genes and Cdk inhibitors, leading to neuronal differentiation and cell-cycle arrest, respectively. Hes1 and Hes5 inhibit this process by directly binding to the promoter of proneural genes, blocking their expression, or by interacting with E proteins, inhibiting the activity of heterodimers. Inhibitor of differentiation (Id) also blocks dimer formation of proneural factors.

Neurogenesis occurs in a specific spatiotemporal manner, so the activity of proneural genes is tightly regulated by various mechanisms. *Inhibitor of differentiation (Id)* genes inhibit the activity of proneural genes by binding to E proteins with a high affinity and form heterodimers that cannot bind to DNA [35, 36]. *Hes* genes block the activity of proneural genes by repressing their transcription or by interfering with complex formation of proneural genes and E-protein [32, 37-39]. At the onset of neurogenesis, different proneural genes are expressed in a sequential manner leading to a process known as the bHLH cascade. Early expression of bHLH transcription factors regulates the late expression of other bHLH genes, for example, paired box 6 (Pax6) activates *Neurog2* expression, which leads to the expression of *Tbr2* and *NeuroD* in intermediate neurogenic progenitor cells (INPs), which result in the induction of *Tbr1* expression in INPs and early differentiating neurons [40, 41].

## 2. Commonly used techniques in brain research

As described above, several factors have been investigated in the context of brain development. However, the exact mechanisms underlying the regulation of each factor or the cross-regulation between different pathways have not been clearly investigated, and different studies have often suggested contradictory results. Among many possible explanations, one cause might be due to the technical drawbacks associated with the currently available brain research models.

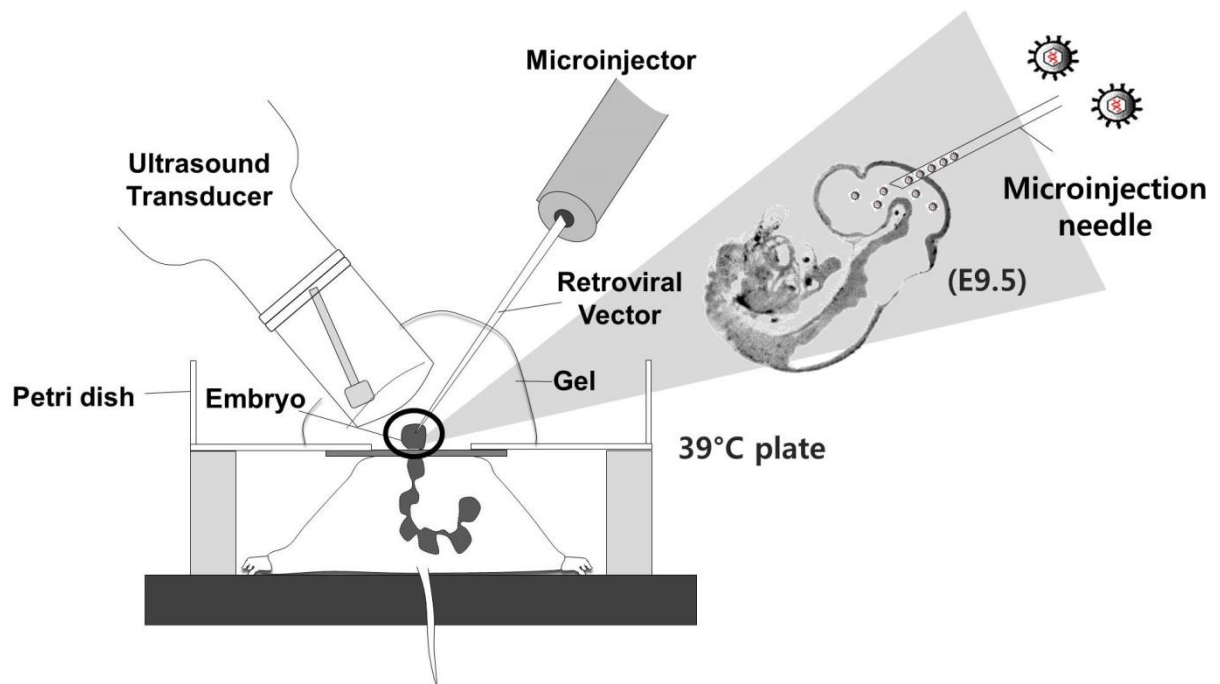
Most *in vivo* studies investigating the effects of various factors on embryonic brain development use mouse models with germline modifications such as transgenic or knock-out (KO) mice. However, this method is not perfectly suitable for studying the specific roles of factors because genetic manipulations affect all cell types and organs in animals, which may cause premature death [42-44]. One can manipulate genes only in a specific type of cell using a cell-specific promoter, but the expression level of the transgene can change and similar genes can compensate for the gene loss over time [45, 46]. Furthermore, when screening of multiple genes is necessary, germline modification is not the best method to proceed as it requires tremendous effort over a long period of time and high maintenance costs.

Another commonly used technique for studying the effects of genes on brain development is *in utero* electroporation, which requires the injection of a plasmid that expresses the gene of interest (GOI) directly into the embryonic brain. However, this method does not permit investigation of the early stages of cell differentiation because the embryo needs to be sufficiently large to enable the identification of the injection target site. Therefore, experiments cannot be performed before E13-14, although neuronal differentiation has already begun by this developmental stage [47].

The ultrasound image-guided gene delivery (UIGD) technique can overcome the limitations of the abovementioned methods [48, 49]. The embryonic brain contains an empty cavity called the forebrain ventricle. While the embryo is monitored by ultrasound imaging, a viral vector expressing the GOI can be injected into this ventricle using a microinjection pipette (Fig. I-4). Gene transfer at the early stage has numerous advantages compared with transfer at E13, which is when *in utero* electroporation is usually performed. Neurogenesis begins at E9–10 [47], and therefore, the early developmental effects of a specific gene can be determined. In addition, more cells can be transduced with the same amount of virus and the expression of the transgene can be maintained by using a retrovirus, which integrates its genome into cellular chromosomes. Most importantly, the UIGD technique is a quick and cost-effective method for producing animals carrying a cerebral transgene, avoiding the compensation effect and alterations in the gene expression level. Given these advantages, the UIGD technique was utilized in the present study to deliver a retrovirus expressing the GOI at E9.5.

### **3. Overview of thesis research**

In this thesis, five stress-related factors were selected for the reasons described in Chapter III, and initially screened to determine their effects on embryonic neurogenesis. Their roles in neuronal differentiation were quickly screened *in vitro* using primary NPCs and confirmed *in vivo*. GSK3 $\beta$  and IFN- $\gamma$  were selected on the basis of their clear negative effects on the neuronal differentiation of NPCs, and further characterizations were performed through various *in vitro* experiments. The results of this



**Figure I-4. Overall procedure of the ultrasound image-guided gene delivery (UIGD) technique.**

The uterus of anesthetized pregnant mice is exteriorized and the fetuses are scanned using an ultrasound biomicroscopic imaging system. A viral vector expressing the GOI is injected into the telencephalic ventricle of E9.5 embryos using ultrasound-guided imaging. The embryos are then transferred back to their original site and grown until analyzed. (Taken from Jang *et al.*, 2013 [50])

study are believed to facilitate the development of preventive drugs or treatment against neurodevelopmental diseases, and may help to understand the crucial steps of neurodevelopment.

## **CHAPTER II**

### **Materials & Methods**

## 1. Cell culture

293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco). NIH3T3 cells were cultured in DMEM supplemented with 10% bovine serum (Gibco). All cells were incubated in 5% CO<sub>2</sub>, 37°C chamber.

## 2. Isolation and *in vitro* culture of mouse neural progenitor cells

All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University. The ganglionic eminences in E14.5 embryonic brains were dissected and washed twice in phosphate buffered saline (PBS). Samples were triturated using a fire-polished Pasteur pipette, and run through a 40-µm cell strainer (Falcon). Cells were cultured in NPC proliferating media, which contains DMEM/F12 (Gibco) with B27 supplement (2% (v/v), 17504-044, Gibco), N2 supplement (1% (v/v), 17502-048, Gibco), Pen Strep Glutamine (1% (v/v), 10378-016, Gibco), 2 µg mL<sup>-1</sup> heparin (H3393, Sigma), 20 ng mL<sup>-1</sup> hFGF basic (100-18B, Pepro Tech), and 10 ng mL<sup>-1</sup> EGF (AF-100-15, Pepro Tech) and incubated in 5% CO<sub>2</sub>, 37°C chamber.

After one day, neurospheres were dissociated into single cells and transduced with retroviral vectors as described below. Two days later, neurospheres were dissociated and seeded on poly-L-ornithine (0.01%, P4957, Sigma) and laminin (10 µg mL<sup>-1</sup>, L2020, Sigma) -coated plates. The cells were incubated in DMEM containing 2% FBS (Gibco) with or without rapamycin (9904, Cell Signaling) and/or SB216763 (S3442, Sigma) dissolved in DMSO. The final concentrations of DMSO were 0.02% (v/v) for rapamycin and 0.01% (v/v) for SB216763.

In experiments with IFN-γ, cells were cultured in new proliferating media for 2 days and differentiated with DMEM containing 2% FBS (Gibco) with or without recombinant mouse IFN-γ (IF005, Millipore), Ruxolitinib (S1378, Selleck) or cycloheximide (Sigma) on poly-L-ornithine (Sigma) and laminin (Sigma)-coated plates.



### 3. Plasmid constructs

The MS vector containing the long terminal repeat from a murine stem cell virus (MSCV) was described previously [51].

The murine GSK3 $\alpha$ , Nrf2, and *Neurog2* promoter without splicing site (#8) were synthesized (Bioneer, Korea). The murine GSK3 $\beta$ , IFN- $\gamma$ , SC4MOL, Neurog2, STAT1, and *Neurog2* promoter sequences [14] were amplified from cDNAs isolated from Raw 264.7, the PMA/Ionomycin stimulated mouse splenocytes, NIH3T3, adult murine hippocampal tissue, HT22 cells, and genomic DNA isolated from murine macrophage cell line (Raw 264.7), respectively using primers listed in Table II-1. The murine NICD sequence was amplified from the plasmid called GNIA [52] as a template. These sequences were cloned into pGEM $\text{\textcircled{R}}$  -T Easy (A1360, Promega) according to the manufacturer's instruction. To construct the mutant GSK3 $\beta$  (Y216F), mutant *Neurog2* promoters, and constitutively active STAT1 (STAT1ca) sequences, site-directed mutagenesis (Stratagene) was employed using pGEM $\text{\textcircled{R}}$  -T Easy-GSK3 $\beta$ , -Neurog2 promoter, and -STAT1 as a template, respectively. Mutant plasmids were confirmed by sequencing. Verified sequences were cloned into the MS-IRES-eGFP, pGL3-Basic (E1751, Promega), or pGL4.17 [luc2/Neo] (E6721, Promega) vector.

The shRNA sequences (Table II-2) targeting murine GSK3 $\alpha$ , GSK3 $\beta$ , STAT1 or luciferase (as the control) were cloned into pSIREN-DsRed (Clontech) vector, which was manipulated by site-directed mutagenesis to contain HA tag. The shRNA sequence targeting luciferase was used as the control.

### 4. Retroviral vectors and transduction procedure

Retroviral vector plasmids were transfected into 293T cells with gag-pol (pCA-gag-pol) and env-expressing vectors (pCA-VSV-G) using Lipofectamine (18324-012, Invitrogen) and PLUS reagent (10964-021, Invitrogen), according to the manufacturer's instructions. Supernatants were

Gene	Primer sequence
Nrf2	F 5'-GGATCC GCCACC ATGATGGATCTGGAGCTGCCCCCTC-3' R 5'-AGATCT TCAATTTTTCTTAGTGTCGGGTT-3'
SC4MOL	F 5'-ACGCGTATGGCCACAAACAAAAGTGTT-3' R 5'-GGATCCTCAATCAGACTTTTTCCCAAG-3'
GSK3 $\beta$ (Y216F)	F 5'-GGAGAGCCCAATGTTTCATTATCTGTTCTCGGTACTAC-3' R 5'-GTAGTACCGAGAACAGATAAATGAAACATTGGGCTCTCC-3'
GSK3 $\beta$	F 5'-AGATCTGCCACCATGTCGGGGCGACCGAGA-3' R 5'-AGATCTTCAGGAAGAGCTAGCGAG-3'
NICD	F 5'-GGATCCGCCACCATGGCTCAGCATGGCCAGCTCTG-3' R 5'-GGATCCCTAAGCGTAATCTGGAACATCGTATGGGTAGCTCGAGCTGTCCAACAGG-3'
IFN- $\gamma$	F 5'-GGATCC GCCACC ATGAACGCTACACACTGCAT-3' R 5'-AGATCT TCAGCAGCGACTCCTTT-3'
Neurog2	F 5'-ACGCGTGCCACCATGTTTCGTCAAATCTGAGACT-3' R 5'-AGATCTCTAGATACAGTCCCTGGCGAG-3'
STAT1	F 5'-ACGCGTGCCACCATGTCACAGTGGTTCGAGCT-3' R 5'-AGATCTTTATACTGTGCTCATCATACTGTCAA-3'
STAT1ca	F 5'-AACTACAAAGTCATGGCTTGTGAGTGTATACCAGAGAATCCCCTG-3' R 5'-CAGGGGATTCTCTGGTATACACTCACAAGCCATGACTTTGTAGTT-3'
Neurog2 promoter #1	F 5'-CCCCTAATGAGCTGCTGAAAGG-3' R 5'-CCATGGATCCTGAGAAAGAAGAGGCAGATG-3'
#2	F 5'-GAGCTCACTGATTGGCCAGCTGCGCCTC-3' R 5'-CCATGGATCCTGAGAAAGAAGAGGCAGATG-3'
#3	F 5'-GAGCTCTCCAGCCGAGCGCGAGCCACCAAT-3' R 5'-CCATGGATCCTGAGAAAGAAGAGGCAGATG-3'
#4	F 5'-GAGCTCGGAGAGGGACTAAAGAAAGGGA-3' R 5'-CCATGGATCCTGAGAAAGAAGAGGCAGATG-3'
#5	F 5'-GAGCTCTTTTCTTGGTGGTATATAAGGGGTT-3' R 5'-CCATGGATCCTGAGAAAGAAGAGGCAGATG-3'
#6	F 5'-CCCCTAATGAGCTGCTGAAAGG-3' R 5'-CCATGGCCTACGCGGCTCCAGATG-3'
#7	F 5'-CCCCTAATGAGCTGCTGAAAGG-3' R 5'-CCATGGCGCCCGAGTCTCGTGTGT-3'

**Table II-1. PCR and mutagenesis primers sequences**

<b>Gene</b>	<b>shRNA sequence</b>
GSK3 $\alpha$ #1	5'–GAAGTGGCTTACACTGACA–3'
GSK3 $\alpha$ #2	5'–GAAGGTTCTTCAGGACAAA–3'
GSK3 $\alpha$ #3	5'–GCTGGACCACTGCAATATTGT–3'
GSK3 $\beta$ #1	5'–GCATGAAAGTTAGCAGAGA–3'
GSK3 $\beta$ #2	5'–GAAAGTTAGCAGAGATAAA–3'
GSK3 $\beta$ #3	5'–GAAAGTGATTGGAAATGGA–3'
STAT1 #1	5'–GGATCAAGTCATGTGCATA–3'
STAT1 #2	5'–TTGCAAGAGCTGAACTATA–3'
STAT1 #3	5'–GCCGAGAACATACCAGAGAAT–3'
Luciferase	5'–GTGCGTTGCTAGTACCAAC–3'

**Table II-2. shRNA sequences**

collected 48 hr after transfection, filtered through a 0.45- $\mu$ m filter and frozen at -80°C until used. Concentrated viral stocks were prepared by ultracentrifugation for 2 hr in a SW32 rotor (Beckman-Coulter) at 20,000 r.p.m. at 4°C. Pellets were resuspended in 50  $\mu$ l of PBS at 4°C overnight, and aliquots of virus were stored at -80°C. For transduction, NIH3T3 cells were seeded at  $1 \times 10^5$  in six-well plates on the previous day. Viral supernatants were added in the presence of polybrene (final concentration 8  $\mu$ g mL<sup>-1</sup>). In the case of mouse primary NPCs, ultracentrifuge-concentrated virus particles were added in the absence of polybrene and incubated at 37°C for 3 hr. After incubation, the cells were washed and cultured in the NPC medium as described above. The viral titer was determined by measuring the percentage of eGFP, dsRED or HA-positive NIH3T3 cells transduced with different dilutions of virus stock. When FACS analysis was needed, the percentages of eGFP<sup>+</sup> cells were analyzed using *FACS Calibur* flow cytometer (BD Biosciences).

## 5. Western blot

NIH3T3 cells or primary NPCs were lysed using RIPA buffer or CytoBuster™ Protein Extraction Reagent (Novagen) with protease and phosphatase inhibitor cocktail. Equal amounts of protein were resolved by 10% (w/v) SDS-PAGE and transferred to PVDF membranes (GE Healthcare). The membranes were blocked with TBST (150 mM NaCl, 10 mM Tris/HCl, 0.1% (v/v) Tween 20, pH 8.0) containing 1% (w/v) BSA (Invitrogen-Gibco) or 5% (w/v) skim milk, and incubated with primary antibodies (listed in Table II-3) diluted in blocking solution at 4°C overnight. The membranes were then incubated with HRP-conjugated goat anti-mouse or rabbit IgG secondary antibody (Sigma) at room temperature (RT) for 1 hr. The bands were visualized with the enhanced chemiluminescence system (WBKLS0500, Millipore), and quantified using Image J software. When needed, blots were stripped using stripping buffer (NCI1059KR, Thermo Scientific) with vigorous shaking for 30 min at RT.

<b>Antibody</b>	<b>Catalogue #</b>	<b>Company</b>	<b>Dilution (IF/IHC/WB)</b>
Rabbit anti-GFP	A11122	Invitrogen	1:500 (IF/IHC)
Rat anti-HA tag	11-867-423-001	Roche	1:300 (IF)
Mouse anti-class III $\beta$ -tubulin (TuJ1)	MMS-435P	Covance	1:500 (IF/IHC)
Mouse anti-Nestin	MAB353	Millipore	1:300 (IF)
Mouse anti-GFAP	MAB3402	Millipore	1:300 (IF/IHC)
Mouse anti-NeuN	MAB377	Millipore	1:50 (IHC)
Rabbit anti-GSK3 $\alpha$	ab28833	Abcam	1:250 (IF)
Mouse anti-GSK3 $\alpha$	sc166116	Santa Cruz	1:100 (WB)
Mouse anti-GSK3 $\beta$	Ab93926	Abcam	1:300 (IF), 1:000(WB)
Alexa Fluor® 488-donkey anti-rabbit IgG	A21206	Invitrogen	1:1000 (IF) 1:500 (IHC)
Alexa Fluor® 488 Donkey Anti-Mouse IgG Antibody	A21202	Invitrogen	1:1000 (IF)
Alexa Fluor® 555-donkey anti-mouse IgG	A31570	Invitrogen	1:1000 (IF) 1:500 (IHC)
Alexa Fluor® 555 Goat Anti-Rat IgG	A21434	Invitrogen	1:1000 (IF)
Alexa Fluor® 488 Goat Anti-Rat IgG	A11006	Invitrogen	1:1000 (IF), 1:500 (IHC)
Hoechst 33258	09460	Polyscience	1:5000 (IF)
Mouse anti-Neurog2	MAB3314	R&D systems	1:100 (WB)
Rabbit anti-total STAT1	9172S	Cell Signaling	1:500 (WB)
Rabbit anti-P-STAT (Tyr701)	9167	Cell Signaling	1:500 (WB)
Mouse anti-phospho-GSK3 (pY216)	612313	BD Bioscience	1:1000 (WB)
Rabbit anti-phospho-GSK3 $\alpha/\beta$ (Ser21/9)	9331	Cell Signaling	1:500 (WB)
Rabbit anti-phospho-p70S6K (Thr389)	9205	Cell Signaling	1:1000 (WB)
Rabbit anti-p70S6K	2708	Cell Signaling	1:1000 (WB)
Mouse anti- $\beta$ -actin	A-5441	Sigma	1:5000 (WB)

**Table II-3. Antibodies used for IF, IHC and Western blot (WB)**

## **6. Quantitative RT-PCR**

Total RNA was isolated using RNAiso Plus (Takara). 1 $\mu$ g of each RNA was used to synthesize cDNAs using Reverse Transcriptase XL (AMV) (Takara) according to the manufacturer's instruction. Quantitative PCR was performed using SYBR Premix Ex Taq (Takara) and the primers listed in Table II-4.

## **7. *In vivo* injection into the ventricle of the embryonic brain**

All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University. Timed pregnant ICR mice (Orient-Bio or Koatech, Korea) were used for viral injections. Virus delivery was performed into the telencephalic ventricle at E9.5 using the ultrasound-guided imaging as described previously [53]. Briefly, pregnant mice were anesthetized with Zoletil 50 (Virbac) and rompun (Bayer Korea). The uterus was exteriorized, and the fetuses were scanned using the ultrasound biomicroscopic imaging system (Vevo660; VisualSonics). Ultracentrifuge-concentrated virus particles were injected into the telencephalic ventricle of E9.5 embryos. Embryos were transferred back into the womb and allowed to grow until E14.5.

## **8. Brain harvest and fixation**

E14.5 brains from embryos were harvested and fixed in 4% paraformaldehyde for 2 hr at 4°C and cryoprotected with 30% sucrose at 4°C until they dropped to bottom. Brains were then frozen in embedding matrix (Cell Path) and stored at -80°C until they were cryosectioned.

## **9. Immunofluorescence, immunohistochemistry assays, and H&E staining**

Immunofluorescence (IF) and immunohistochemistry (IHC) assays were performed using the primary and secondary antibodies listed in Table 3. For IF, cells were fixed with 4%

Gene	Primer sequence
<i>Neurog2</i>	F 5'–AACTCCACGTCCCCATACAG–3' R 5'–GAGGCGCATAACGATGCTTC–3'
<i>Math1</i>	F 5'–GTAAGGAGAAGCGGCTGTG–3' R 5'–AGCCAAGCTCGTCCACTA–3'
<i>Mash1</i>	F 5'–GCAACCGGGTCAAGTTGGT–3' R 5'–GTCGTTGGAGTAGTTGGGGG–3'
<i>Tbr2</i>	F 5'–GGCCCCTATGGCTCAAATTC–3' R 5'–CCTGCCCTGTTTGGTGATG–3'
<i>IRF1</i>	F 5'–AGAGGAACCAGAGATTGACAGC–3' R 5'–GTCAGAGACCCAAACTATGGTGC–3'
<i>SC4MOL</i>	F 5'–AAACAAAAGTGTTGGCGTGTTTC–3' R 5'–AAGCATTCTTAAAGGGCTCCTG–3'
<i>GAPDH</i>	F 5'–CTGGAAAGCTGTGGCGTGAT–3' R 5'–CCAGGCGGCACGTCAGATCC–3'

**Table II-4. qRT-PCR primer sequences**

paraformaldehyde, permeabilized with PBS containing 0.5% Triton X-100, and then blocked for 1 hr with PBS containing 10% FBS. The samples were incubated first with primary antibodies diluted in blocking solution overnight at 4°C, and then incubated with secondary antibodies diluted in PBS for 1 hr at room temperature. Hoechst staining was done with 1  $\mu\text{g mL}^{-1}$  Hoechst 33258 (09460, polyscience). For IHC assay, sections were washed in PBS, and then blocked for 1 hr with PBS containing 1% FBS and 0.2% Triton X-100. Sections were incubated first with primary antibodies diluted in blocking solution overnight at 4°C, and then incubated with secondary antibodies diluted in blocking solution for 1 hr at room temperature. Hoechst staining was done with 1  $\mu\text{g mL}^{-1}$  Hoechst 33342. Hematoxylin and eosin (H&E) staining was done by Histopathology Core Lab in Seoul National University (Seoul, Korea).

The stained cells or sections were visualized and their images were acquired using Zeiss (Oberkochen, Germany) LSM 510 or LSM700 confocal microscope. Cells were counted using Image J software. Signal intensity was quantified using ZEN 2012 (blue edition) software.

## **10. Migration assay**

E14.5 primary NPCs were transduced with the control or GSK3 $\beta$ -expressing retroviral vectors. After 2 days, eGFP-positive cells were sorted using FACS Aria III (Becton Dickinson).  $1.25 \times 10^5$  of sorted cells suspended in 100  $\mu\text{L}$  of F12 DMEM were plated on PLO and laminin coated 8.0  $\mu\text{m}$  polycarbonate membrane. The lower chamber contained 600  $\mu\text{L}$  of 2% FBS in F12 DMEM with 100 ng/mL SCF (255-sc, R&D systems). After 1 day, the upper surface of the membrane was washed, and the membrane was fixed with 4% PFA for 30 min at RT and stained with crystal violet for 30 min at RT.

E14.5 primary NPCs were allowed to form neurospheres and were allowed to differentiate with or without IFN- $\gamma$  for 1 day. Cells were fixed, permeabilized, blocked and stained with TuJ1-specific antibody as described above. The distance from the edge of the neurosphere to the furthest



outgrowing cell was measured and normalized to the diameter of the neurosphere using Image J software.

## **11. TUNEL assay**

Cells were fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.5% Triton X-100, and then blocked for 1 hr with PBS containing 10% FBS. TUNEL staining was performed using the in situ Cell Death Detection Kit TMR red (Roche), according to the manufacturer's instructions. The stained cells were visualized and their images were acquired using Zeiss (Oberkochen, Germany) LSM 510 or LSM 700 confocal microscope. Cells were counted using Image J software.

## **12. MTT assay**

NPCs were differentiated with various concentrations of IFN- $\gamma$  for 2 days. Cell viability was analyzed using MTT assay kit (Roche) according to the manufacturer's protocol.

## **13. Luciferase reporter assay**

pNeurog2-Luc or pNeurog2 $\Delta$ -Luc with p $\beta$ -gal were co-transfected into NIH3T3 cells using Lipofectamine and PLUS reagent (Invitrogen), according to the manufacturer's protocol. After 3 hr, fresh media was added with or without 50 ng/mL IFN- $\gamma$ . NPCs were transfected with pNeurog2-Luc or pNeurog $\Delta$ -Luc, using Neon<sup>®</sup> Transfection System (Invitrogen) (1500V, 10ms, 3pulses). After 1 day, cells were differentiated with or without 50 ng/mL IFN- $\gamma$  in DMEM containing 2% FBS for 12 hr. All cells were lysed with Reporter Lysis Buffer (Promega) and the firefly luciferase assay reagent was added to equal amounts of cell lysates. Transfection efficiency was measured using  $\beta$ -galactosidase assay. The luminescent signal was measured using a microplate luminometer.

For stable integration, Neurog2pro-Luc-Neo<sup>R</sup> was linearized with *Bam*HI. A total of  $1.6 \times 10^6$  NIH3T3 cells were suspended in 400  $\mu$ L of serum-free medium mixed with 10  $\mu$ g of linearized Neurog2pro-Luc-Neo<sup>R</sup> plasmid, and subjected to electroporation using Gene Pulser Xcell electroporation system (220V, 975  $\mu$ F, Bio-Rad) followed by G418 selection (25  $\mu$ g/ml). G418-resistant cell clone with the highest basal luciferase activity was selected, expanded, and subjected to luciferase reporter assay as described above.

## **14. Statistical analysis**

All values are presented as mean  $\pm$  standard deviation (SD) from three independent experiments. Differences between values were determined by a one-way ANOVA followed by Tukey's post-hoc test or two-way ANOVA, using GraphPad Prism software (Version 5, GraphPad Software). Differences in the signal intensity from IF assays and the band intensity from Western blot analysis were determined by unpaired t-test and paired t-test, respectively using GraphPad Prism software. P-values less than 0.05 were considered to be statistically significant.

## **CHAPTER III**

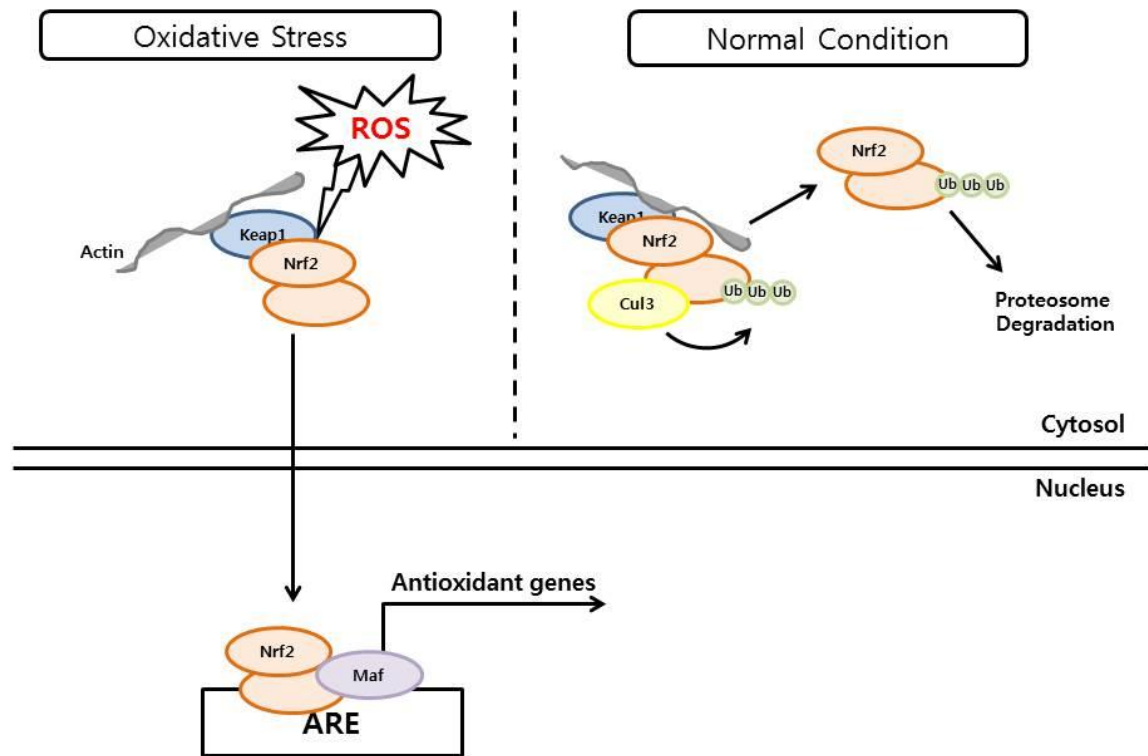
# **Effects of Stress-related Genes on Embryonic Neurogenesis**

# 1. Background

The most prevalent risk factors that can lead to serious brain defects are alcohol consumption and infection during pregnancy. Children of mothers who have been exposed to alcohol during pregnancy exhibit an increased risk of mental retardation, microcephaly, and functional brain problems such as memory loss and cognitive defects [2, 54-56]. Similarly, children who experienced an infection during their fetal development have a higher risk of various brain defects such as microcephaly, schizophrenia, autism, cerebral palsy, and mental retardation [57-62]. In this chapter, five candidate factors (Nrf2, GSK3 $\alpha$ , GSK3 $\beta$ , IFN- $\gamma$ , and SC4MOL) which have increased expression levels during fetal alcohol exposure or prenatal infection were selected, and their effects on the initiation of neurogenesis were investigated.

## 1.1 Rationale for choosing Nrf2

The Nrf2-antioxidant response element (ARE) pathway is a well-known pathway in the antioxidant system, which is activated upon oxidative stress such as alcohol exposure that generates reactive oxygen species (ROS) in the mother's womb and the fetus [63]. When oxidative stress occurs, transcription factor Nrf2 translocates to the nucleus and regulates the expression of numerous proteins that contribute to the antioxidant response such as heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (Nqo1), and glutamate-cysteine ligase (Fig.III-1). Alcohol consumption induces the generation of ROS in both the mother and fetus, which leads to an approximately three-fold increase in Nrf2 expression in the fetus [64, 65]. Despite its expression during embryonic development, the role of Nrf2 in fetal development has been elusive. Nrf2 KO mice exhibit no developmental or reproduction deficits; therefore, the role of Nrf2 in development has not been studied extensively. However, a study published in 2003 using a double KO mouse line for Nrf2 and Nrf1, both of which belong to the same cap 'n' collar type of basic region leucine zipper factor (CNC-bZip) family, suggested that members of the CNC-bZip family might compensate for each other as the double KO



**Figure III-1. Schematic representation of Nrf2 signaling pathway.** In normal conditions, Nrf2 is constitutively expressed and bound by actin-anchored protein Kelch-like ECH associating protein 1 (Keap1), which is an adaptor protein of Cullin-3-based E3 ubiquitin ligase complex (Cul3). Cul3 continuously ubiquitinates Nrf2 and leads to its proteosomal degradation. In the presence of oxidative stress, the redox-sensitive cysteine residue (C273 and 288) of Keap1 is oxidized, resulting in a conformational change and the release of Nrf2. The free Nrf2 then translocates into the nucleus and forms a heterodimer with Maf protein. This complex binds to ARE and regulates the expression of antioxidant genes.

mice have a shorter *in utero* life compared with the Nrf1 KO mice [66]. Based on this finding, several studies have investigated the roles of Nrf2 in cell differentiation and development; however, these are at an early stage [67, 68].

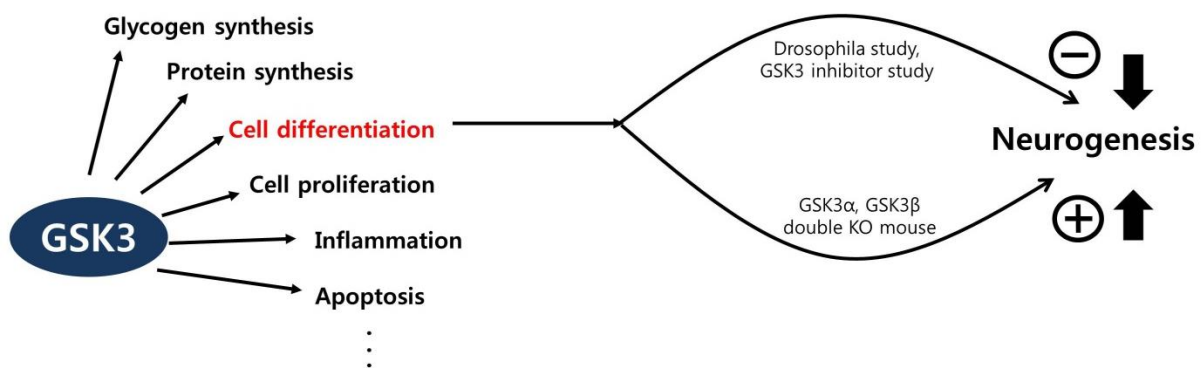
## 1.2 Rationale for choosing GSK3

GSK3 is a serine/threonine kinase, which is involved in numerous physiological processes including glucose metabolism, apoptosis, cellular proliferation, and differentiation. GSK3 has two isoforms, GSK3 $\alpha$  and GSK3 $\beta$ , which share 98% homology in their kinase domains. It is involved in cellular division and differentiation, but its role in the differentiation of NPCs is controversial (Fig. III-2). Several *in vitro* studies have been performed using chemical inhibitors that bind to the ATP binding pocket of GSK3 to block its kinase activity. Studies using these GSK3 inhibitors showed that GSK3 inhibits neurogenesis. However, chemical inhibitors cannot distinguish between the two isoforms of GSK3; therefore, the specific role of each isoform has not been determined. In addition, these experiments were conducted only *in vitro*.

*In vivo* investigation of the two isoforms began after GSK3 transgenic animals became available. In contrast to the results obtained using inhibitors, GSK3 $\alpha$  or GSK3 $\beta$  single KO mice do not show any significant cerebral defects. However, hyperproliferation and decreased differentiation of NPCs are detected in the brains of GSK3 $\alpha/\beta$  double KO mice. Therefore, the precise roles of the GSK3 isoforms in embryonic neurogenesis are unclear and controversial, and thus, functional investigation of GSK3 isoform is necessary.

## 1.3 Rationale for choosing IFN- $\gamma$

In infants or children, congenital viral infections can lead to severe neurological diseases such as blindness, epilepsy, hydrocephalus, and mental retardation; these in turn can increase the risk of schizophrenia and autism [57-62]. Fetal brain damage might be induced by the pathogen itself; however, it has been suggested that the activated immune response may be the main cause of this



**Figure III-2. Contradictory results regarding the role of GSK3 in NPC differentiation.** GSK3 is involved in various cellular processes, but the exact roles of each isoform are unclear. No major brain malformations have been found in GSK3 $\alpha$  KO mice or in mice engineered to have the GSK3 $\beta$  gene deleted in their NPCs, whereas the deletion of both the GSK3 $\alpha$  and GSK3 $\beta$  genes induces hyperproliferation and suppresses neuronal differentiation. By contrast, a *Drosophila* mutant with a defective *shaggy* gene, a GSK3 homologue, exhibits increased neuronal differentiation. Use of GSK3 inhibitors also promotes neuronal differentiation of human NPCs, rat ventral midbrain precursors, and rat neural stem cells.

pathogenesis [69]. IFN- $\gamma$  is a critical cytokine released by host immune cells upon infection, but despite its significant roles in the host immune response, the effects of IFN- $\gamma$  on fetal brain development has not been clearly understood.

Several studies have been performed to determine the function of IFN- $\gamma$  in NPC differentiation; however, different results were reported depending on the experimental conditions (Fig.III-3). In addition, the downstream mechanisms regulated by IFN- $\gamma$  have not been clarified, and there have been no *in vivo* results related to its function in the embryonic brain. In this thesis, the effects of IFN- $\gamma$  on brain development were investigated using both *in vitro* and *in vivo* experimental systems.

## **1.4 Rationale for choosing SC4MOL**

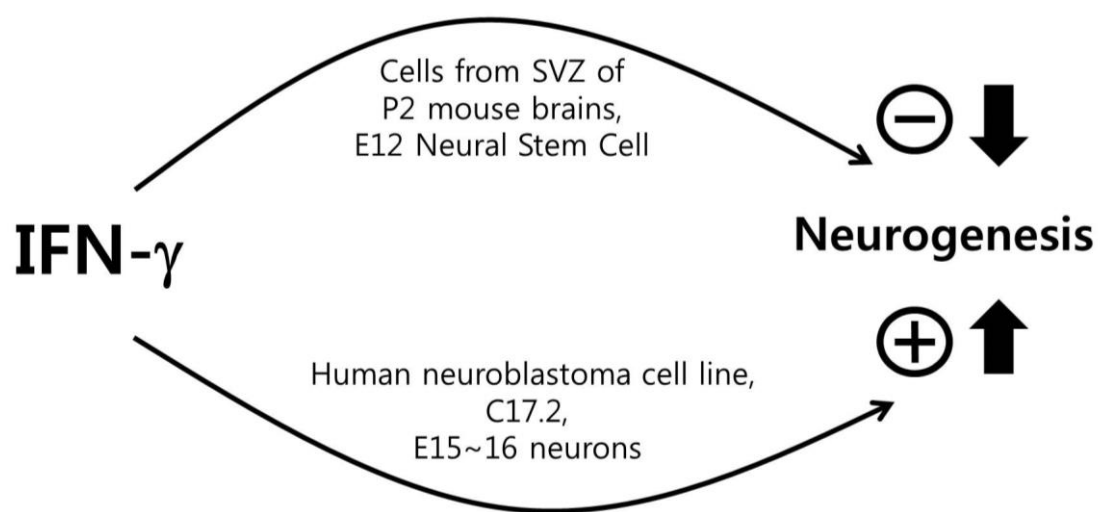
SC4MOL is involved in cholesterol synthesis. Defects in cholesterol synthesis can lead to severe symptoms such as developmental defects or neonatal lethality, but no study has addressed the relationship between SC4MOL and brain development. In 2011, a case report showed that a patient with psoriasiform dermatitis, arthralgia, congenital cataracts, microcephaly, and developmental delay possessed two point mutations in the SC4MOL gene (519T  $\rightarrow$  A and 731A  $\rightarrow$  G) [70]. A study published in 2012 reported that the expression level of SC4MOL was increased in the fetal brain in three maternal immune activation (MIA) models [71]. Based on these results, SC4MOL was chosen to investigate whether it has an effect on the differentiation of NPCs.

## **2. Results**

### **2.1 Strategy for studying candidate genes**

In order to investigate the role of each factor in the most efficient manner, an *in vitro* screening method was employed using mouse primary NPCs. To perform gain of function studies, retroviral vectors that express the GOI and the enhanced green fluorescent protein (eGFP) reporter





**Figure III-3. Contradictory results regarding the role of IFN- $\gamma$  in NPC differentiation.** IFN- $\gamma$  induced neurogenesis in experiments involving a human neuroblastoma cell line, neonatal NPC line (C17.2), and E15-E16 neurons. However, IFN- $\gamma$  obtained the opposite result with cells from the subventricular zone of postnatal day 2 mouse brains, and E12 neural stem cells. SVZ, subventricular zone.

gene in a bicistronic message were created. The expression and activity of each gene was tested, and E14.5 NPCs were then transduced with the respective viral vectors and differentiated for 3 days (Fig. III-4).

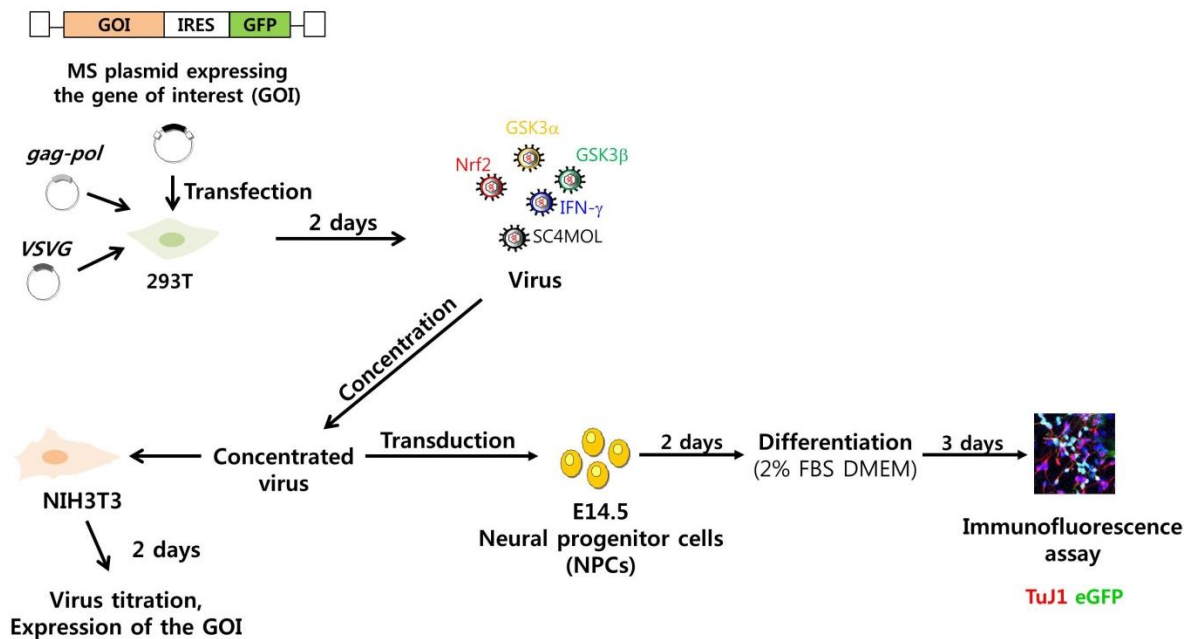
## **2.2 Effects of Nrf2 on embryonic neurogenesis**

To test the role of Nrf2 in NPC differentiation, a retroviral bicistronic vector that expressed both Nrf2 and eGFP was generated. The viral construct was transduced into NIH3T3 cells, and the expression of exogenous Nrf2 was determined by western blotting using an Nrf2-specific antibody (Fig. III-5A). Nrf2 was not detectable in the control cells, whereas it was detected in Nrf2-overexpressing cells. The activity of exogenous Nrf2 was determined by measuring the expression level of HO-1, a downstream target of Nrf2. As expected, the level of HO-1 increased by approximately four-fold in cells transduced with the Nrf2-expressing retroviral vector.

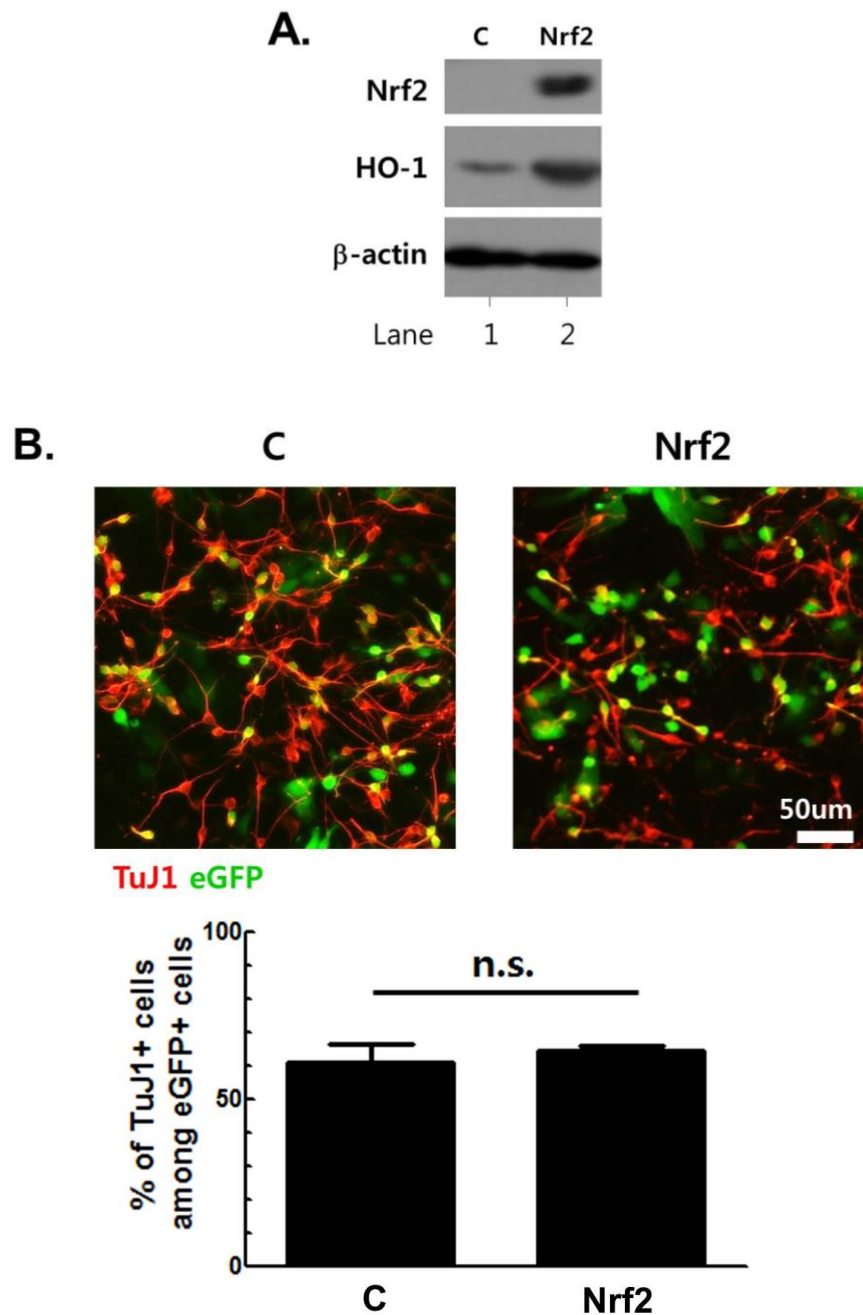
Primary NPCs were transduced with the retroviral vector expressing Nrf2, and differentiation was then induced for 3 days. Among the cells transduced with the control vector that only expresses eGFP, approximately 60% of the eGFP-positive cells were positive for the neuron-specific TuJ1. A similar ratio of TuJ1-positive cells was observed in the Nrf2-transduced cells, indicating that Nrf2 does not influence neuronal differentiation of NPCs (Fig. III-5B).

## **2.3 Effects of the GSK3 isoforms on embryonic neurogenesis**

To examine the roles of GSK3 isoforms in neuronal differentiation, the effect of GSK3 overexpression was investigated. NIH3T3 cells were transduced with retroviral vectors expressing GSK3 $\alpha$  or GSK3 $\beta$  together with eGFP, and the expression levels of the isoforms were determined using western blotting. Endogenous GSK3 $\alpha$  was not detectable, while endogenous GSK3 $\beta$  was visible only after longer exposures in the control cells. Exogenous GSK3 $\alpha$  and GSK3 $\beta$  were detected when



**Figure III-4. Overall procedure employed for *in vitro* screening of factors that affect neuronal differentiation of NPCs.** Retroviral vector plasmids expressing the GOI and eGFP in a bicistronic message were transfected into 293T cells with *gag-pol* (pCA-*gag-pol*) and *env*-expressing vectors (pCA-VSV-G). Supernatants were collected at 48 hr after transfection and concentrated by ultracentrifugation. The viral titre and the expression of the GOI were analyzed by transducing NIH3T3 cells with concentrated virus particles in the presence of polybrene. To determine the effects of each gene on the differentiation of NPCs, concentrated virus particles were added to E14.5 NPCs in the absence of polybrene. After 3 days, the cells were differentiated with DMEM containing 2% FBS and immunostained with antibodies specific to eGFP and neuron-specific TuJ1.



**Figure III-5. Effects of Nrf2 on the neuronal differentiation of NPCs.** (A) Retroviral vectors expressing Nrf2 together with eGFP were added to NIH3T3 cells for 2 days. The total cell lysates were subjected to western blotting using antibodies to Nrf2 and HO-1. (B) NPCs were transduced with the same titer of respective retroviral vectors and then induced to differentiate for 3 days, followed by immunostaining for eGFP with TuJ1. Representative images of the staining and the percentages of each cell type among the eGFP-positive cells are shown. Scale bar, 50  $\mu$ m. n.s., not significant; C, control.

cells were transduced with the respective retroviral vectors, and tyrosine phosphorylated GSK3, which is an indication of GSK3 activity, was also detected (Fig. III-6A).

Primary NPCs were transduced with retroviral vectors expressing GSK3 $\alpha$  or GSK3 $\beta$  at the same titer, and then differentiated for 3 days. Among the cells transduced with the control vector expressing eGFP only, approximately 60% of the eGFP-positive cells were positive for TuJ1. A similar ratio of TuJ1-positive cells was observed in GSK3 $\alpha$ -transduced cells, while this ratio was reduced to 20% in GSK3 $\beta$ -transduced cells (Fig. III-6B). These results indicate that GSK3 $\beta$ , but not GSK3 $\alpha$ , inhibits neuronal differentiation.

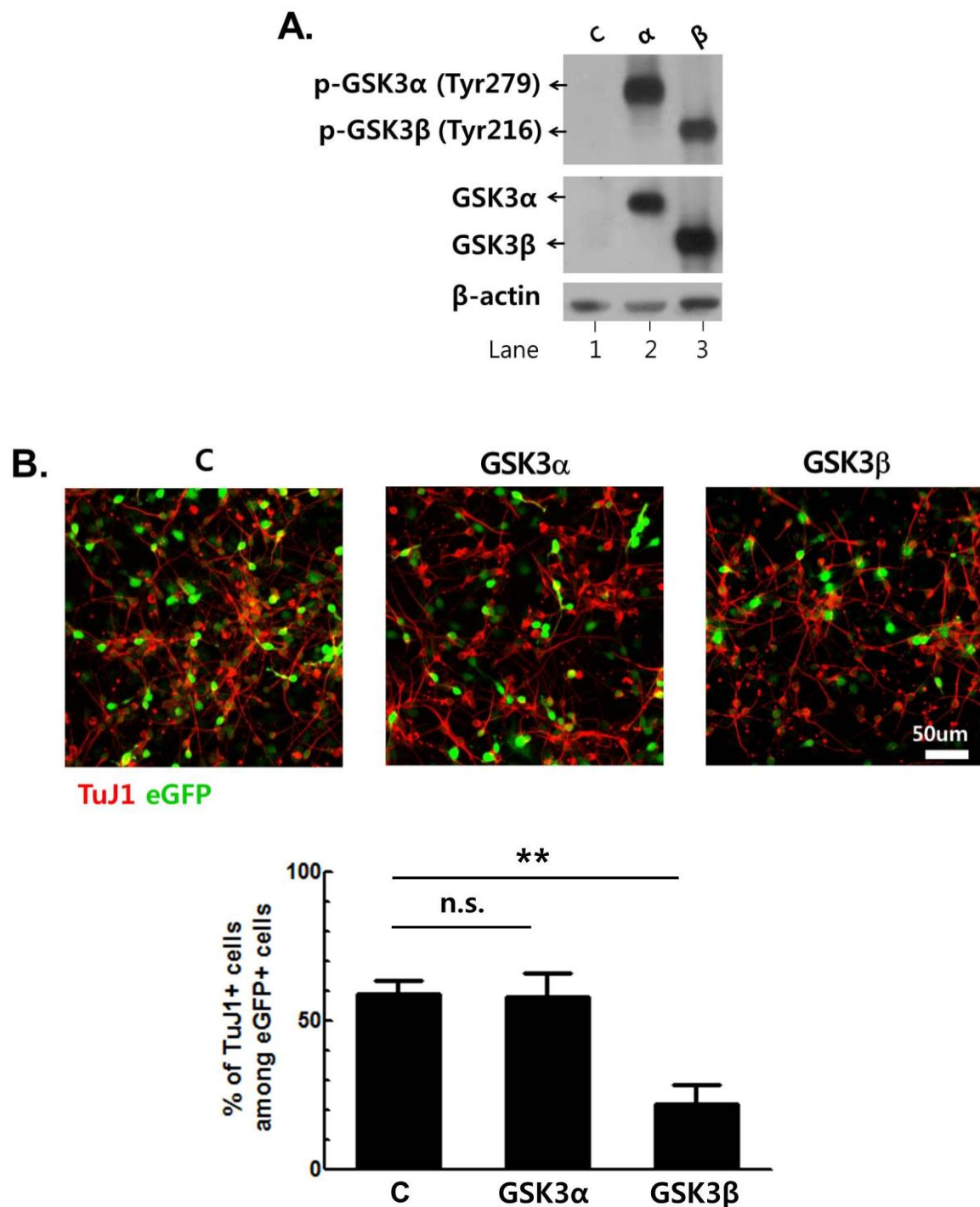
## **2.4 Effects of IFN- $\gamma$ on embryonic neurogenesis**

Similar to the aforementioned constructs, an IFN- $\gamma$ -expressing retroviral vector was generated and transduced into primary NPCs. IFN- $\gamma$  was not detected in the control cells, while approximately 10 ng/mL of IFN- $\gamma$  was secreted in the transduced NPCs (Fig. III-7A). Tyrosine-phosphorylated STAT1 was also detected, suggesting that the exogenous IFN- $\gamma$  is functional (Fig. III-7B).

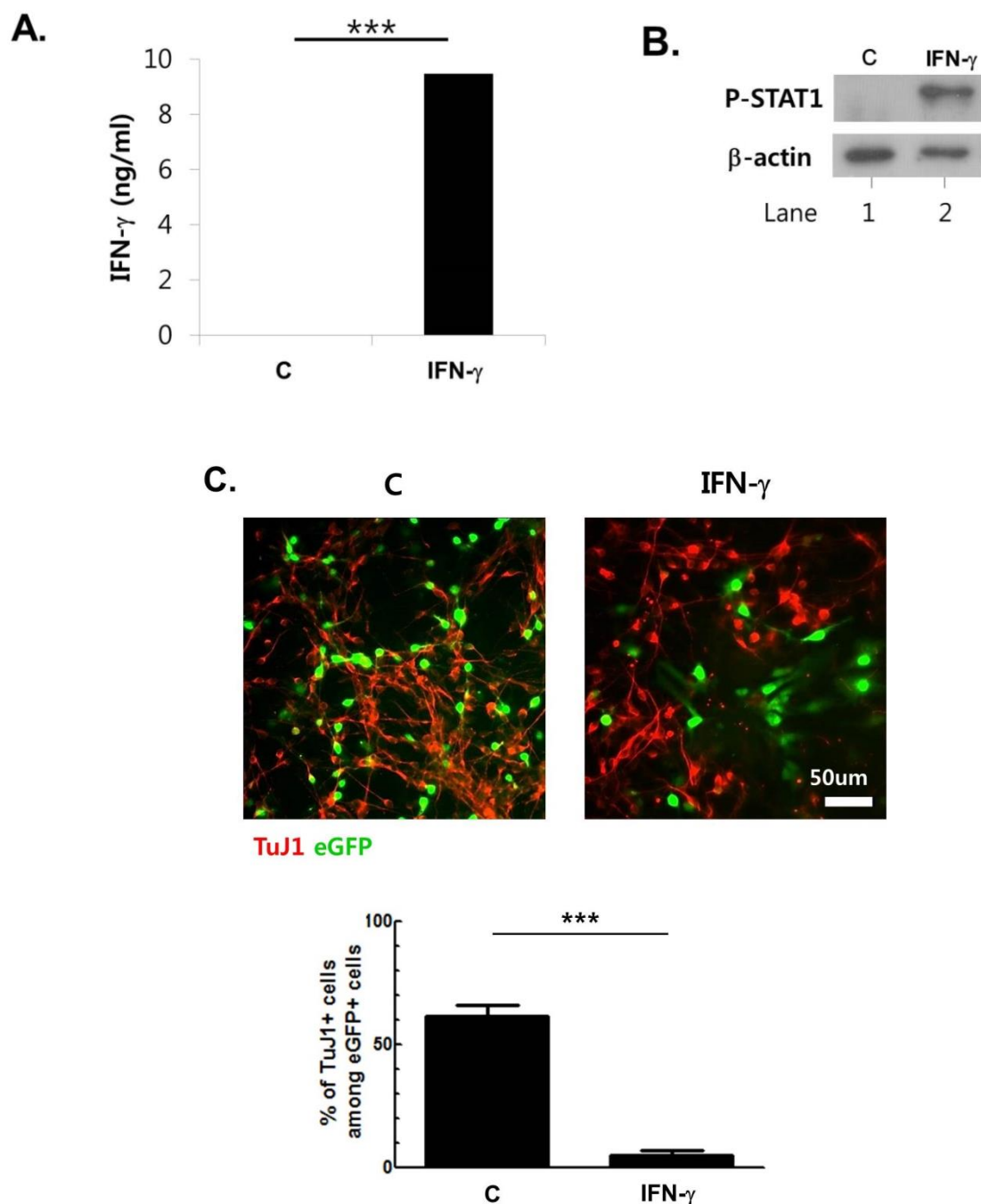
When primary NPCs were transduced with a retroviral vector that expresses IFN- $\gamma$  and differentiated, the ratio of TuJ1-positive cells dramatically decreased by approximately 70% compared with the cells transduced with the control construct (Fig. III-7C). This result demonstrates that IFN- $\gamma$  can strongly inhibit neuronal differentiation.

## **2.5 Effects of SC4MOL on embryonic neurogenesis**

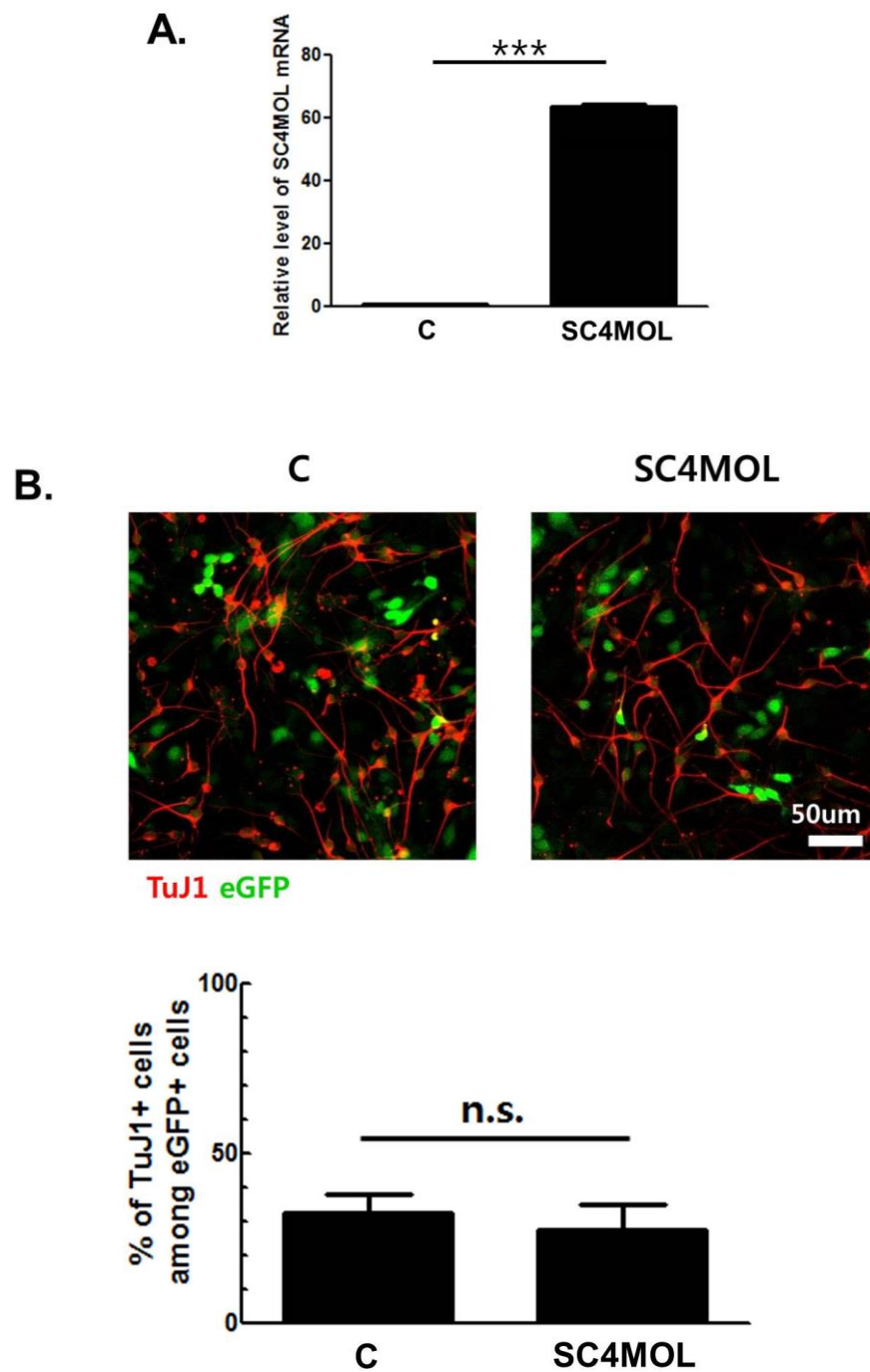
Primary NPCs were transduced with a retroviral vector expressing SC4MOL. The expression level of SC4MOL was increased by more than 63-fold compared with the control cells (Fig. III-8A). When SC4MOL-expressing NPCs were differentiated for 3 days, there was no statistically significant difference in the ratio of TuJ1-positive neurons between the two groups (Fig. III-8B), suggesting that



**Figure III-6. Effects of GSK3 isoforms on the neuronal differentiation of NPCs.** (A) Retroviral vectors expressing GSK3 $\alpha$  or GSK3 $\beta$  together with eGFP were added to NIH3T3 cells for 2 days. Total cell lysates were subjected to western blotting using antibodies to total GSK3 isoforms and tyrosine phosphorylated GSK3 isoforms. (B) NPCs were transduced with the same titer of respective retroviral vectors and then induced to differentiate for 3 days, followed by immunostaining for eGFP with TuJ1. Representative images of the staining and the percentages of each cell type among the eGFP-positive cells are shown. Scale bar, 50  $\mu$ m. \*\*P < 0.01; n.s., not significant; C, control.



**Figure III-7. Effects of IFN- $\gamma$  on the neuronal differentiation of NPCs.** (A,B) Retroviral vectors expressing IFN- $\gamma$  together with eGFP were added to NPCs for 2 days. Supernatants and cell lysates were prepared and subjected to ELISA and western blotting using antibodies to IFN- $\gamma$ , P-STAT1 (Tyr701) and  $\beta$ -actin. (C) NPCs were transduced with the same titer of respective retroviral vectors and then induced to differentiate for 3 days, followed by immunostaining for eGFP with TuJ1. Representative images of the staining and the percentages of each cell type among the eGFP-positive cells are shown. Scale bar, 50  $\mu$ m. \*\*\* $P < 0.001$ ; C, control.



**Figure III-8. Effects of SC4MOL on the neuronal differentiation of NPCs.** (A) Retroviral vectors expressing SC4MOL together with eGFP were added to NPCs for 2 days. qRT-PCR was performed using SC4MOL-specific primers. (B) NPCs were transduced with the same titer of respective retroviral vectors and then induced to differentiate for 3 days, followed by immunostaining for eGFP with TuJ1. Representative images of the staining and the percentages of each cell type among the eGFP-positive cells are shown. Scale bar, 50  $\mu$ m. \*\*\* $P < 0.001$ ; n.s., not significant; C, control



SC4MOL does not play an important role in embryonic neurogenesis.

### 3. Discussion

In this chapter, a rapid screening system using retroviral vectors and NPCs was employed to efficiently screen important factors in the context of embryonic neurogenesis. The factors used in this study were previously reported as being upregulated in the prenatally stressed embryonic brain, but their effects on neurogenesis have not been studied in the context of NPC differentiation. In experiments involving the overexpression of Nrf2, GSK3 $\alpha$ , and SC4MOL, the number of differentiated neurons was comparable with that in the control group, while this number was dramatically reduced when GSK3 $\beta$  or IFN- $\gamma$  was overexpressed. The results of these experiments suggest that Nrf2, GSK3 $\alpha$ , and SC4MOL do not play crucial roles in embryonic neurogenesis, whereas GSK3 $\beta$  and IFN- $\gamma$  strongly inhibit it. Gain-of-function system employed in this study have limitations as the endogenous expression of each gene might already be maximal, producing no additional effects when the level of expression is increased further. However, these data provided useful insights that can be used to conduct further investigations.

The process of neuronal differentiation is complex and only a small number of genes have been clearly demonstrated to be involved in the regulation of neurogenesis. The links between various signaling pathways have not been well understood and the underlying events during development that can result in serious neurological disorders are elusive. Thus, improved understanding of brain science and studies using NPCs may provide necessary information to facilitate the development of suitable therapies for patients with congenital/acquired brain disorders. If the factors involved in the differentiation of NPCs can be clearly identified and the nature of NPC regulation is further characterized, it will lead to a deeper understanding of brain pathology and possibly open up new approaches for the prevention or treatment of many brain disorders. According to the results described in this chapter, GSK3 $\beta$  and IFN- $\gamma$  are two factors that appeared to have negative effects on the

neuronal differentiation of NPCs. Thus, they were further characterized using various molecular techniques as described in the next two chapters.

## **CHAPTER IV**

### **Roles of GSK3 Isoforms in Embryonic Neurogenesis**

# 1. Background

GSK3 is a serine/threonine kinase that plays pivotal roles in many physiological processes including glucose metabolism, cell survival, proliferation, and differentiation [72]. In mammals, there are two isoforms of GSK3, GSK3 $\alpha$  and GSK3 $\beta$ , that share 98% homology in their kinase domain [73]. Although it is clear that GSK3 is involved in the proliferation and differentiation of NPCs [74], the exact roles of each isoform have not been clearly defined. No major brain malformations are found in GSK3 $\alpha$  KO mice or in mice engineered to have the GSK3 $\beta$  gene deleted in their NPCs [75], while the deletion of both the GSK3 $\alpha$  and GSK3 $\beta$  genes induces hyperproliferation and suppresses neuronal differentiation [76]. By contrast, mutant *Drosophila* that are defective in the *shaggy* gene, a GSK3 homologue, show increased neuronal differentiation [77]. The use of GSK3 inhibitors also promotes neuronal differentiation of human NPCs, rat ventral midbrain precursors, and rat neural stem cells [78-80]. Due to the conflicting results of these studies, the functions of GSK3 in the differentiation of NPCs and the exact effects of GSK3 $\alpha$  and GSK3 $\beta$  have been elusive.

GSK3 exists in cells in a constitutively active form, and its kinase activity is regulated by phosphorylation. The activity of GSK3 is downregulated when serine 21 of GSK3 $\alpha$  and serine 9 of GSK3 $\beta$  are phosphorylated, and upregulated when tyrosine residues (tyrosines 279 and 216 of GSK3 $\alpha$  and GSK3 $\beta$ , respectively) are phosphorylated [81]. Many kinases and phosphatases, such as protein kinase B (PKB), MAPK-activated protein kinase 1 (MAPKAP-K1), protein phosphatase (PP) 1, and PP2A, are known to influence the activity of GSK3 [82-84]. The mammalian target of rapamycin complex1 (mTORC1) is a kinase that has been reported to be involved in the serine phosphorylation of GSK3. In LPS-stimulated monocytes and tuberous sclerosis complex (TSC) 1 or TSC2 mutant embryonic fibroblasts, rapamycin has been found to block the serine phosphorylation of GSK3 $\beta$  [85, 86]. Although the relationship between GSK3 and mTORC1 is well defined in various situations such as cancer and inflammation, this relationship has not been delineated in the context of NPC differentiation. The activation of mTORC1, induced by insulin treatment or deletion of the TSC1 gene in embryonic telencephalic NPCs, results in premature differentiation, and rapamycin (an mTORC1

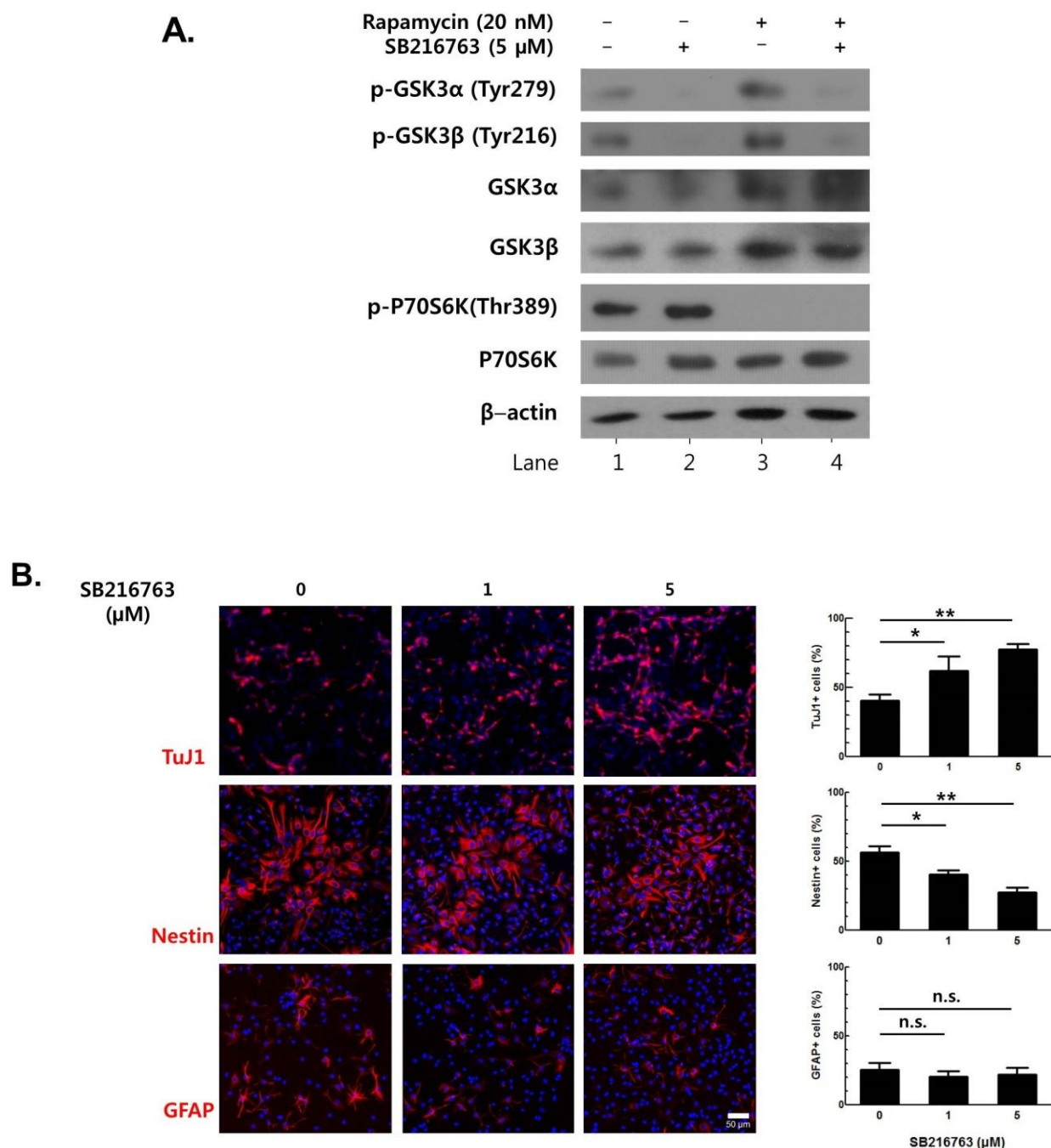
inhibitor) treatment inhibits these effects; these results suggest that mTORC1 has a role in neuronal differentiation [87, 88]. However, the downstream mechanism of mTORC1 in neurogenesis remains to be determined.

In this chapter, data obtained from *in vitro* and *in vivo* experiments involving E14.5 NPCs and E9 embryonic brains are described. These results indicate that GSK3 $\beta$  inhibits NPC differentiation. In addition, downregulation of GSK3 $\beta$  was found to de-repress the rapamycin-mediated inhibition of neuronal differentiation. Overall, these findings suggest that GSK3 $\beta$ , but not GSK3 $\alpha$ , is the isoform that negatively affects the differentiation of NPCs as the downstream target of the mTORC1 signaling pathway.

## **2. Results**

### **2.1 Effects of GSK3 inhibition on the neuronal differentiation of NPCs**

In several previous studies, chemical inhibitors of GSK3 have been used in attempts to define the roles of GSK3 [78-80]. To confirm the effect of GSK3 inhibition on the differentiation of NPCs, SB216763, an ATP-competitive inhibitor of GSK3 was used [89]. Primary NPCs were prepared from E14.5 mouse embryos and differentiated with DMEM containing 2% FBS and treated with 5  $\mu$ M SB216763 for 3 days. The effects of SB216763 on the tyrosine phosphorylation (an indicator of active GSK3) of each isoform [74, 90, 91] were observed by western blotting (Fig. IV-1A, compare lanes 1 and 2). The level of tyrosine phosphorylation of both GSK3 $\alpha$  and GSK3 $\beta$  were decreased 3 hr after 5  $\mu$ M SB216763 treatment, indicating the decreased activity of GSK3. Differentiated cells were then stained with specific markers including antibodies to TuJ1, Nestin (NPCs), and GFAP (astrocytes). Upon treatment with 5  $\mu$ M SB216763, the percentage of TuJ1-positive cells increased from 40% to 77%, but the percentage of Nestin-positive cells decreased from 57% to 28% (Fig. IV-1B). GFAP-positive cells were also counted to determine the effect of GSK3 on the formation of astrocytes, and no significant effect was observed. These data are consistent with

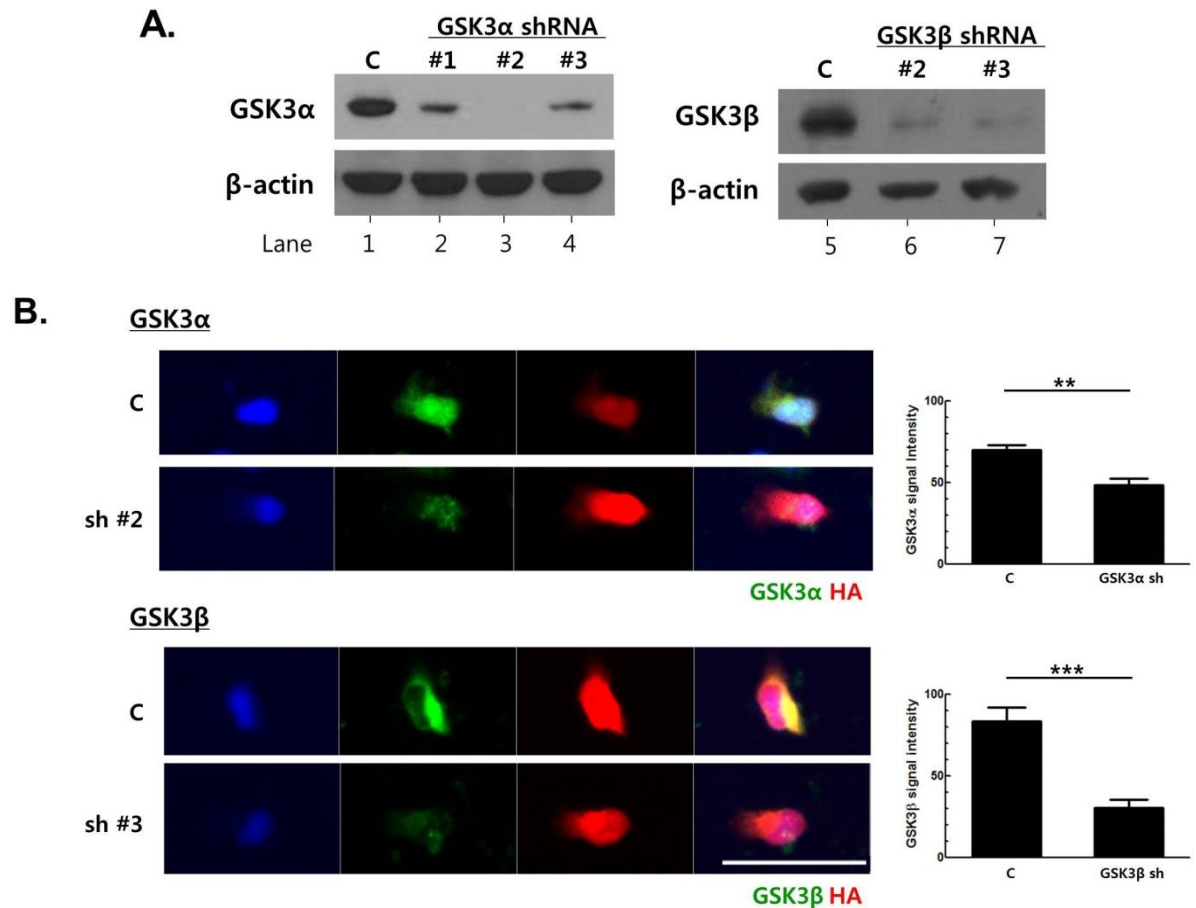


**Figure IV-1. Inhibition of GSK3 isoforms increases the neuronal differentiation of primary NPCs.** (A) Primary NPCs were induced to differentiate in the presence of 5 mM SB216763 and 20 nM rapamycin, separately or together, for 3 hr. Total cellular proteins were prepared and subjected to western blot. All proteins were analyzed using the same blot. (B) Primary NPCs were induced to differentiate with Dulbecco's modified Eagle's medium containing 2% fetal bovine serum in the presence of SB216763 for 3 days, followed by immunostaining for TuJ1, Nestin, and GFAP. Representative images of the staining and the percentages of each cell type are shown.

previous results in that the inhibition of both GSK3 $\alpha$  and  $\beta$  isoforms via chemical inhibitors enhanced the neuronal differentiation of progenitor cells, suggesting that GSK3 has a negative effect on neurogenesis.

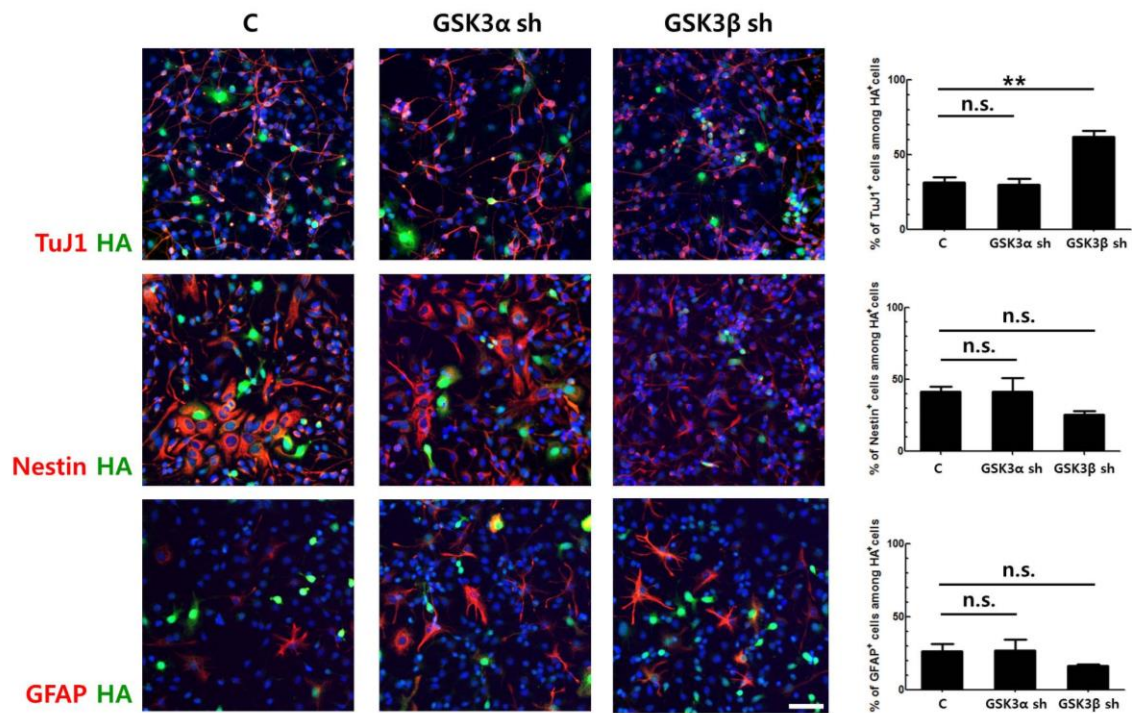
To investigate which isoform of GSK3 contributes to the inhibition of neuronal differentiation, retroviral vectors expressing three different shRNA sequences against GSK3 $\alpha$  or GSK3 $\beta$ , together with HA tagged dsRED, were constructed. To determine knock-down efficiency, GSK3 $\alpha$ -overexpressing NIH3T3 cells and NIH3T3 cells were transduced with retroviral vectors expressing GSK3 $\alpha$  or GSK3 $\beta$  shRNA, respectively. GSK3 $\alpha$ -overexpressing NIH3T3 cells were used because the basal expression level of GSK3 $\alpha$  in NIH3T3 cells was undetectable. When the protein level of each isoform was measured after 2 days using Western blot, it was found that GSK3 $\alpha$  shRNA sequence #2 (Fig. IV-2A, lane 3) and GSK3 $\beta$  shRNA sequence #3 (Fig. IV-2A, lane 7) produced the highest knock-down efficiencies ( $98.9 \pm 1.5\%$  and  $71.3 \pm 10.9\%$  reduction compared to the control, respectively). To be certain, knock-down efficiency was also tested using primary NPCs. Cells were transduced with a retroviral vector expressing respective shRNAs, and 2 days later, the protein level was analyzed by immunostaining (Fig. IV-2B). When signal intensity was measured, the protein level of GSK3 $\alpha$  and GSK3 $\beta$  was decreased by  $30.6 \pm 12.7\%$  and  $63.5 \pm 15.2\%$ , respectively.

To investigate the effect of downregulation of GSK3 $\alpha$  and GSK3 $\beta$  on cell differentiation, primary NPCs were transduced with the same titer of the retroviral vectors expressing respective shRNAs and then induced to differentiate for 2 days. As DsRed fluorescence was too weak to be identified using a fluorescent microscope, the HA tag was stained, and the percentages of different cell markers among HA-positive cells were calculated (Fig. IV-3). In cells transduced with a control vector, approximately 31% of the transduced cells were TuJ1-positive. Interestingly, the number of TuJ1-positive cells among cells transduced with GSK3 $\alpha$  shRNA-expressing vector was similar to that of controls, while this number increased by approximately 2-fold in the GSK3 $\beta$  shRNA-expressing cells. Similarly, the proportion of Nestin-positive NPCs was decreased by nearly half when the expression of GSK3 $\beta$  was knocked-down. The changes in the percentages of GFAP-positive cells



**Figure IV-2. Knock-down efficiency of GSK3 shRNAs.** (A) GSK3 $\alpha$ -overexpressing NIH3T3 cells and NIH3T3 cells were transduced with retroviral vectors expressing GSK3 $\alpha$  shRNA and GSK3 $\beta$  shRNA, respectively. The knock-down efficiency of each shRNA sequence was analyzed by western blotting, using antibodies to GSK3 $\alpha$  and GSK3 $\beta$ . (B) Primary NPCs were transduced with retroviral vectors expressing GSK3 $\alpha$  shRNA No. 2 and GSK3 $\beta$  shRNA No. 3 for 2 days. The knock-down efficiency of each shRNA sequence was confirmed by immunostaining, using antibodies to HA tag with GSK3 $\alpha$  or GSK3 $\beta$ . The signal intensity of GSK3 $\alpha$  or GSK3 $\beta$  was quantified by ZEN 2012 (blue edition) software.





**Figure IV-3. Downregulation of GSK3 $\beta$ , but not GSK3 $\alpha$ , increases the neuronal differentiation of primary NPCs.** Primary NPCs were transduced with the same titer of respective retroviral vectors and then induced to differentiate for 2 days, followed by immunostaining for HA tag with TuJ1, Nestin, or GFAP. Representative images of the staining and the percentages of each cell type among HA-positive cells are shown. DNA was labeled with Hoechst 33258. Scale bar, 50  $\mu$ m. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; n.s., not significant; C, control.

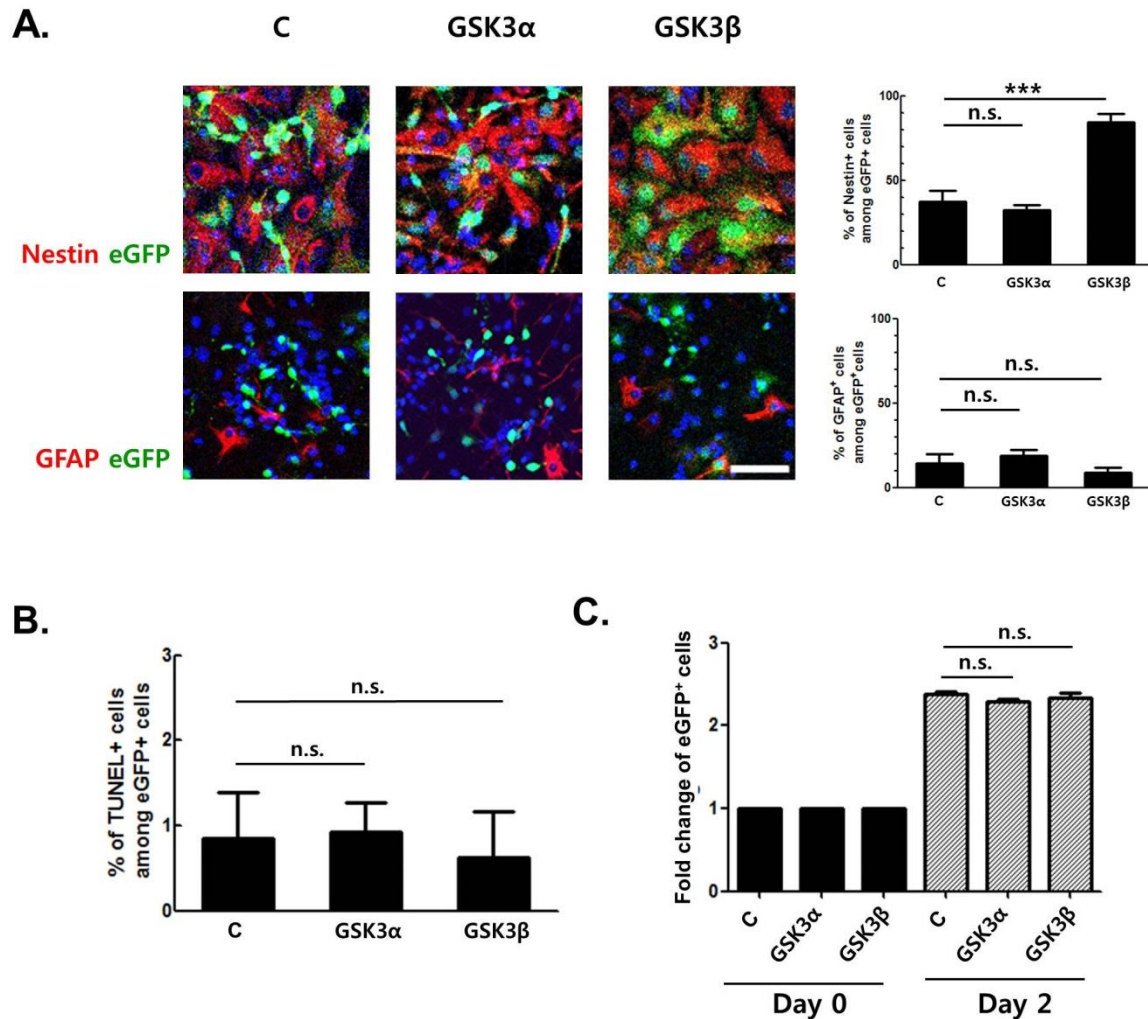
were not statistically significant. These data suggest that GSK3 $\alpha$  and GSK3 $\beta$  might have different effects on neuronal differentiation.

## **2.2 Effects of GSK3 overexpression on the differentiation and proliferation of NPCs**

In the previous chapter, it was demonstrated that overexpression of GSK3 $\beta$  inhibits the neuronal differentiation of NPCs. To further characterize the role of GSK3 isoforms in NPC differentiation, the percentage of other cell types including NPC and astrocyte were analyzed. Similar to the control, approximately 40% of the GSK3 $\alpha$ -transduced cells were positive for Nestin. However, the constitutive expression of GSK3 $\beta$  increased the percentage of Nestin-positive cells to 80%. The numbers of GFAP-positive cells were similar in all three groups (Fig.IV-4A).

To test whether the decrease in the percentage of TuJ1-positive cells resulted from NPC death, apoptotic cell death was analyzed. NPCs were transduced with GSK3 $\alpha$ - and GSK3 $\beta$ -overexpressing vectors for 2 days and differentiated for 3 days. The cells were then subjected for a TUNEL assay. As shown in Fig. IV-4B, the numbers of TUNEL-positive cells were comparable between the control, GSK3 $\alpha$ -, and GSK3 $\beta$ -transduced cells, indicating that apoptosis did not have a significant influence.

The effect of GSK3 isoforms on the proliferation of NPCs was also examined by FACS analysis. Primary NPCs were transduced with respective retroviral vectors, and 2 days later, changes in the number of GFP<sup>+</sup> NPCs were measured (Fig.IV-4C). In all three groups, the number of GFP<sup>+</sup> cells was doubled to a similar level by day 2, and there was no significant difference between the control and GSK3 $\alpha$ - and GSK3 $\beta$ - overexpressing cells. These data indicate that GSK3 does not influence the proliferative potential of NPCs. Taken together, abovementioned results corroborate the notion that GSK3 $\beta$ , but not GSK3 $\alpha$ , inhibited neuronal differentiation and helped NPCs to maintain their stemness.

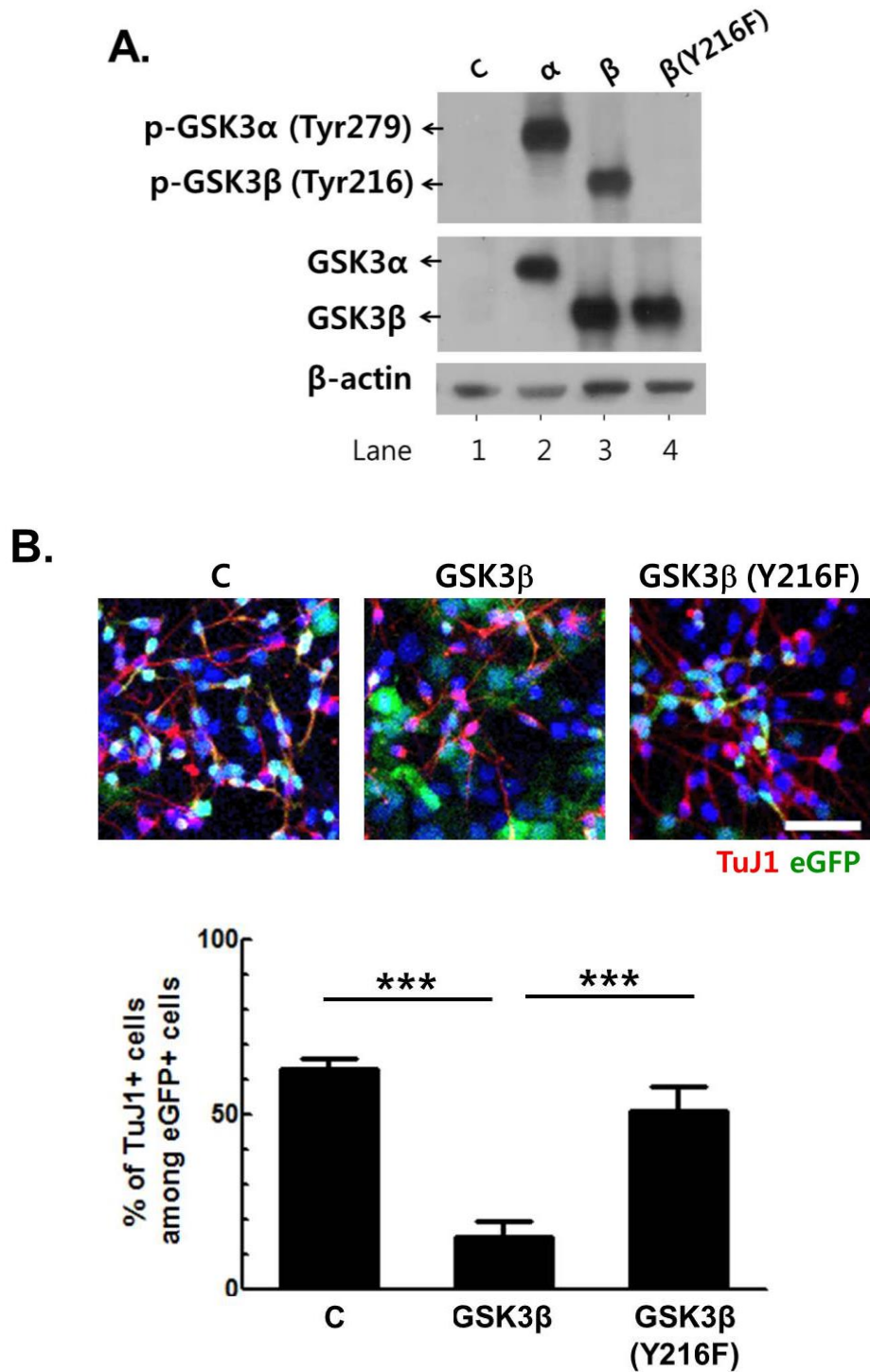


**Figure IV-4. GSK3 $\beta$ , but not GSK3 $\alpha$ , suppresses the neuronal differentiation of NPCs without affecting cellular apoptosis and NPC proliferation.** (A) Primary NPCs were transduced with the same titer of respective retroviral vectors and then induced to differentiate for 3 days. The cells were subjected to immunostaining for eGFP and Nestin, or GFAP. Representative images of the staining and the percentages of each cell type among the eGFP-positive cells are shown. (B) Two days after the transduction of primary NPCs with the above retroviral vectors, they were subjected to differentiate for 3 days, and apoptotic cell death was analyzed by a TUNEL assay. (C) Two days after the transduction of primary NPCs with above retroviral vectors, the % of GFP+ cells were analyzed by fluorescence-activated cell sorting for 2 days.

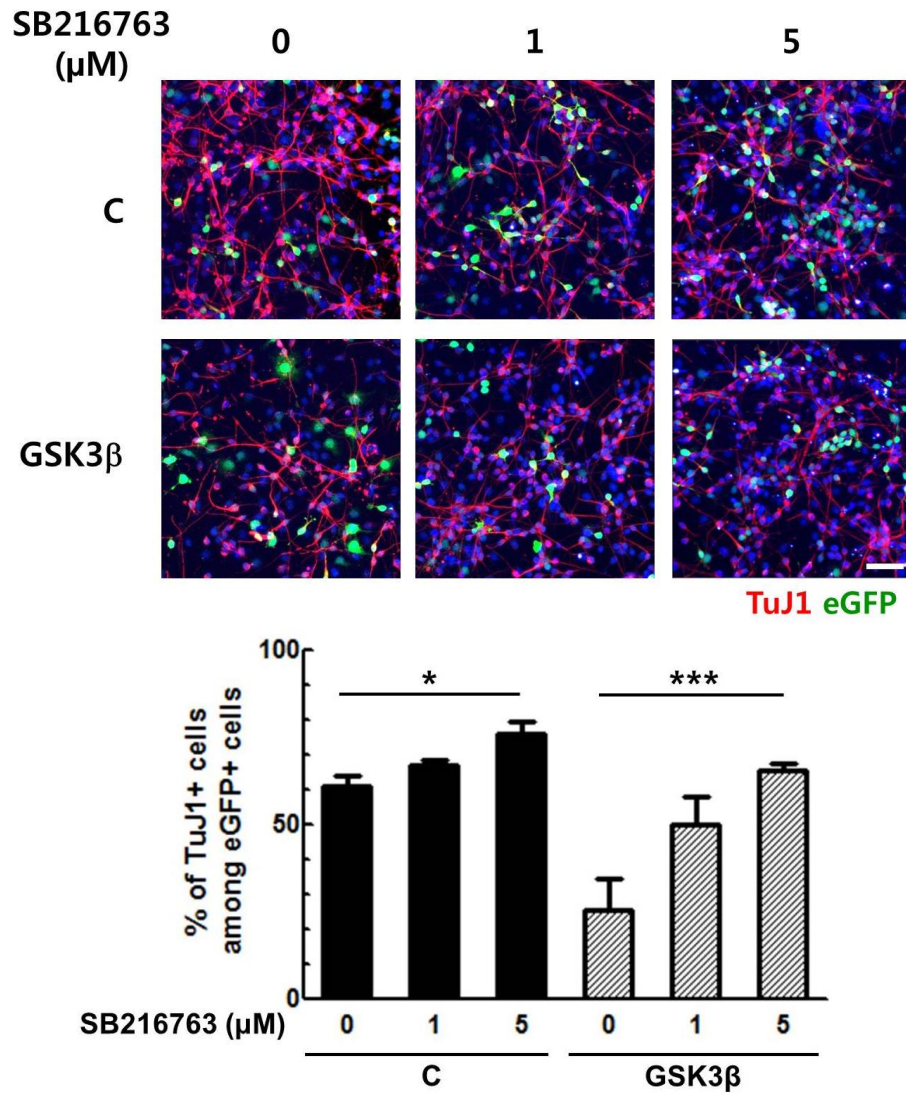
To determine whether the kinase activity of GSK3 $\beta$  is involved in the inhibition of neuronal differentiation, a retroviral vector expressing a mutant form of GSK3 $\beta$  that contains phenylalanine in the place of tyrosine at the 216<sup>th</sup> codon was constructed. This mutant form has previously been shown to exhibit reduced kinase activity [90], and its expression was confirmed by western blotting as shown in Fig. IV-5A (lane 4). Primary NPCs were transduced with retroviral vectors expressing the wild type or this mutant sequence of GSK3 $\beta$  and induced to differentiate for 3 days. Retroviral expression of this mutant form resulted in a significantly greater number of TuJ1-positive cells compared to cells expressing the wild-type protein (Fig. IV-5B). When GSK3 $\beta$ -expressing progenitor cells were induced to differentiate in the presence of SB216763 for 3 days, the GSK3 $\beta$ -mediated inhibition of neuronal differentiation was abrogated in a dose-dependent manner (Fig. IV-6). These data demonstrate that GSK3 $\beta$  negatively controls the neuronal differentiation of progenitor cells and that the kinase activity of GSK3 $\beta$  is essential to this inhibition.

## **2.3 Effects of GSK3 $\beta$ on neuronal differentiation in the embryonic brain**

To test the effect of GSK3 $\beta$  on neuronal differentiation during brain development *in vivo*, a retroviral vector expressing GSK3 $\beta$  and eGFP as a bicistronic message was delivered to the ventricles of E9.5 embryonic brains using the UIGD technique [53], and the brains were analyzed at E14.5. Under this experimental condition, NPCs in the VZ are transduced with retroviral vectors, and the neurons produced from these NPCs migrate away from the VZ during neurogenesis. Because the NICD is well known to strongly inhibit neuronal differentiation [52, 92], this sequence was used as a positive control. When a control vector expressing only eGFP was injected into embryonic brains, approximately 86% of eGFP-positive cells were found in the TuJ1-stained region, while NICD-transduced cells were predominantly localized in the VZ (Fig. IV-7A and B). In the case of GSK3 $\beta$ , approximately 31% of cells were positive for TuJ1, and this percentage was significantly lower than that of the control. These data suggest that GSK3 $\beta$  can indeed suppress neuronal differentiation and

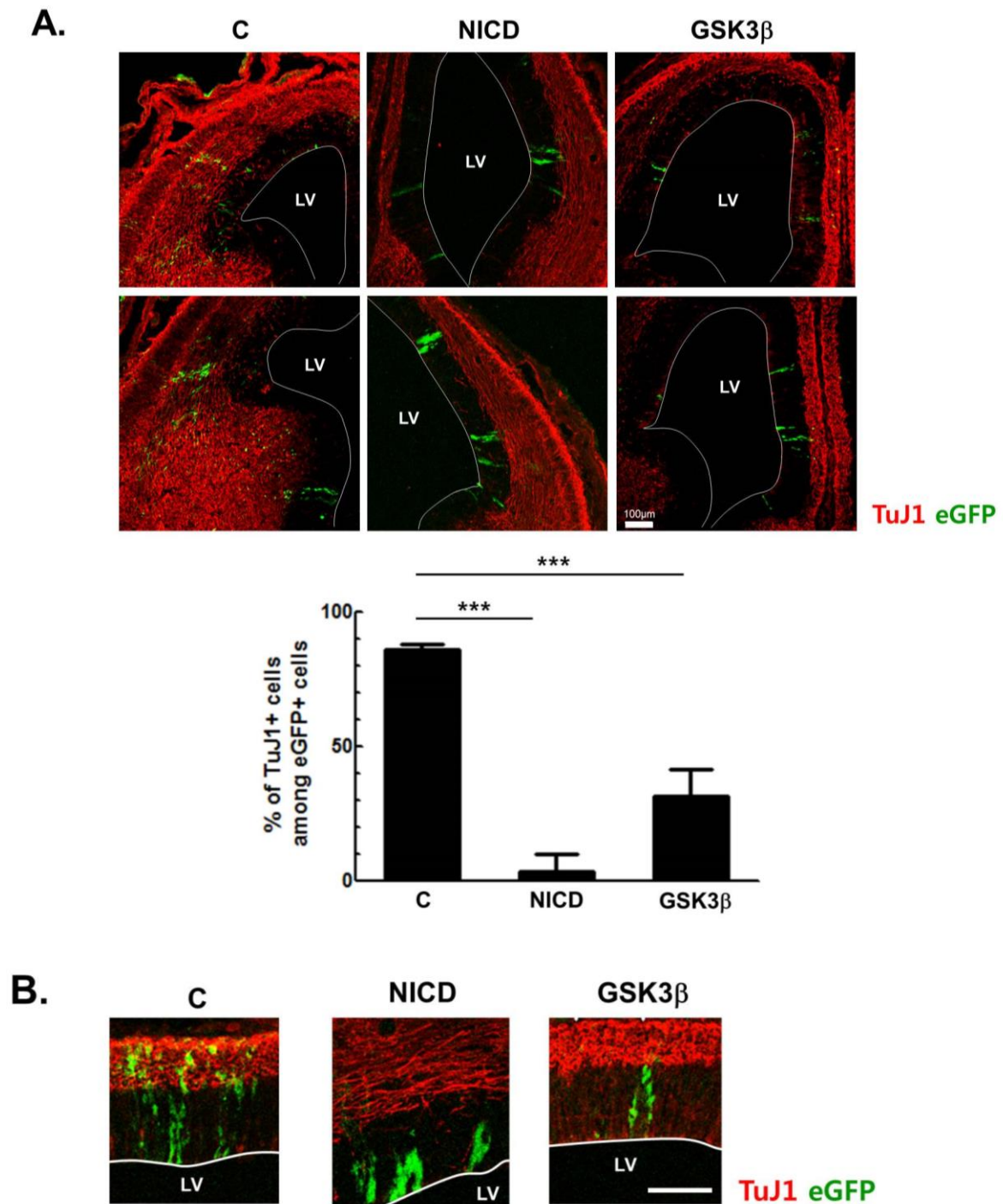


**Figure IV-5. The kinase activity of GSK3β is required for the suppression of neurogenesis. (A)** Retroviral vectors expressing GSK3α, GSK3β, or GSK3β (Y216F), together with eGFP, were used to transduce NIH3T3 cells. The level of expression and tyrosine phosphorylation of GSK3α, GSK3β, or GSK3β(Y216F) was analyzed by western blotting, using specific antibodies. **(B)** Retroviral vectors expressing wild-type GSK3β or mutant GSK3β (Y216F) were used to transduce primary NPCs, followed by differentiation for 3 days. Representative images of eGFP and TuJ1 staining are shown.



**Figure IV-6. GSK3 $\beta$  negatively controls the neuronal differentiation of NPCs.** Primary NPCs were transduced with a retroviral vector expressing GSK3 $\beta$ , and then induced to differentiate for 3 days in the presence of SB216763. The cells were double labeled with antibodies to eGFP and TuJ1. The percentages of TuJ1-positive cells among the eGFP-positive cells are presented. DNA was labeled with Hoechst 33258. Scale bar, 50  $\mu$ m. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001; n.s., not significant; C, control.





**Figure IV-7. GSK3 $\beta$  inhibits neuronal differentiation *in vivo*.** Retroviral vectors expressing Notch1 intracellular domain (NICD) or GSK3 $\beta$ , together with eGFP, were injected into E9.5 embryonic brains ( $n = 3/\text{group}$ ), and then the brains were immunostained with antibodies to eGFP and TuJ1 at E14.5. **(A)** Representative images of sections from two embryonic brains are shown. Among the eGFP-positive cells, the number of TuJ1-positive cells was counted in coronal sections through the lateral ventricle (LV). Results from the cell counting are shown. **(B)** Enlarged images.

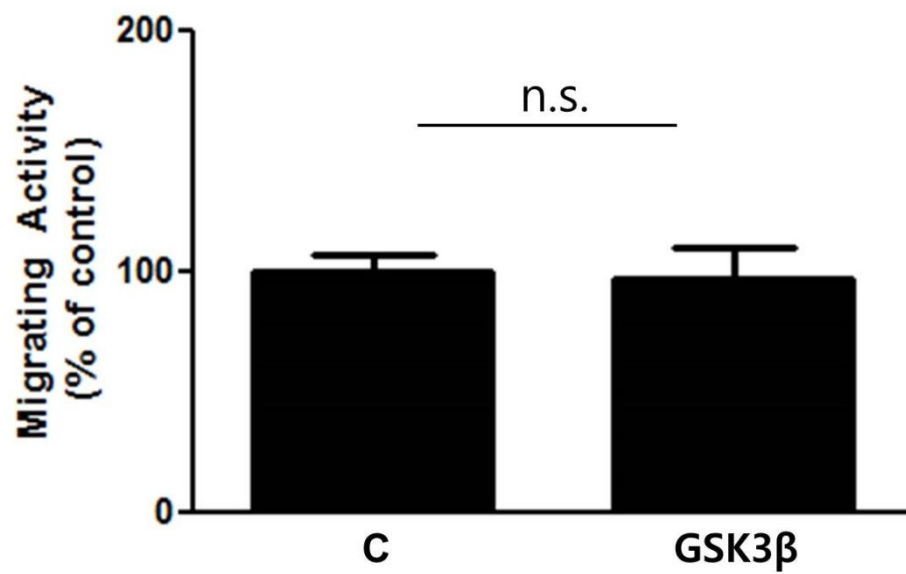
that it was involved in maintaining the characteristics of NPCs in embryonic brains.

To determine whether the retention of NPCs in the VZ in the GSK3 $\beta$  group resulted from the inhibition of cell migration, the effects of GSK3 $\beta$  overexpression on the migration of NPCs were also tested. Primary NPCs were transduced with the control or GSK3 $\beta$ -expressing retroviral vectors. After 2 days, eGFP-positive cells were sorted and migrating ability was measured by transwell migration assay (Fig. IV-8). There was no statistically significant difference between the control and GSK3 $\beta$ -overexpressing cells, suggesting that GSK3 $\beta$  does not affect the migrating ability of NPCs.

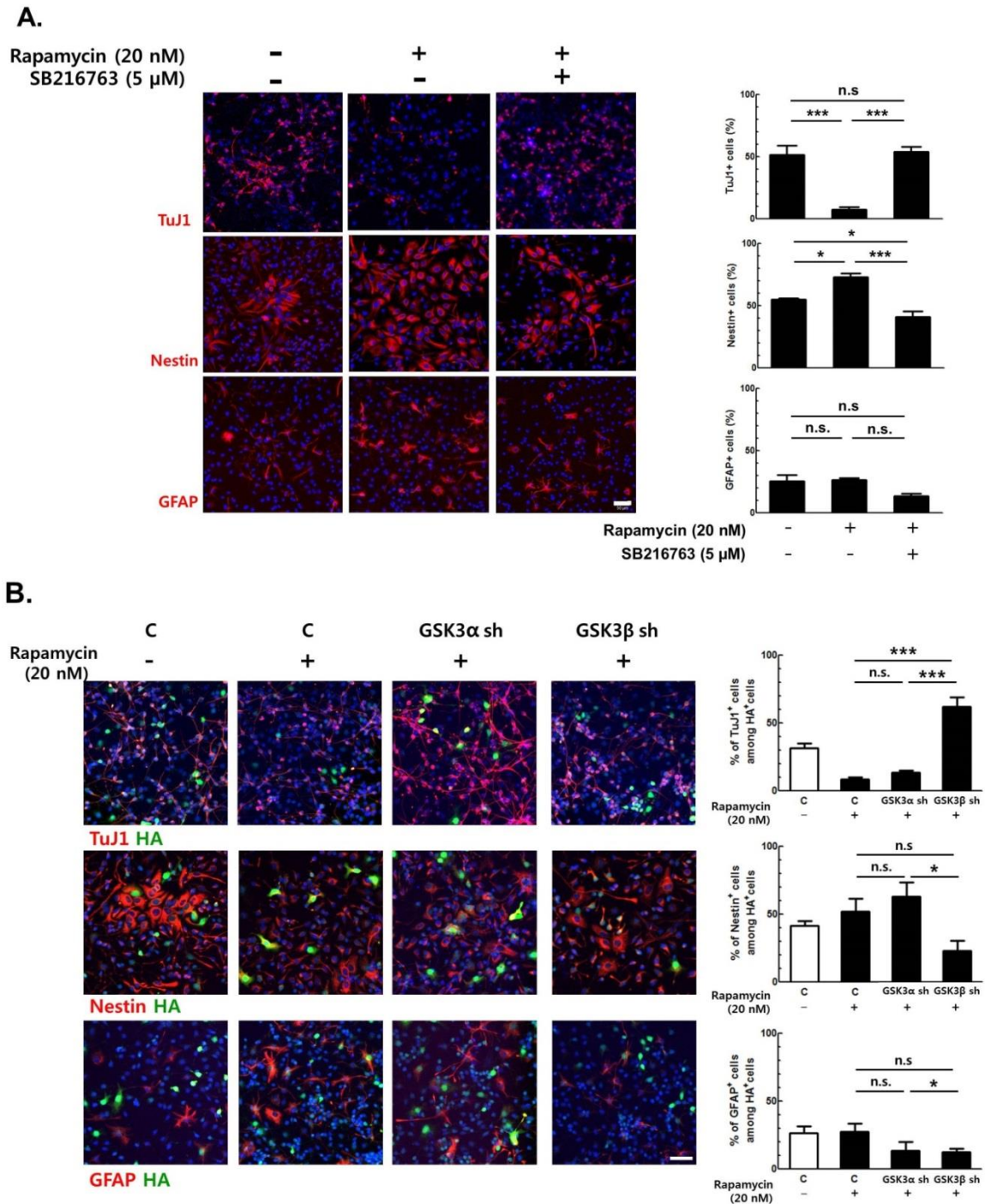
## **2.4 Regulation of GSK3 by the mTORC1 signaling pathway**

Rapamycin, an mTORC1 inhibitor, has been reported to repress the neuronal differentiation of NPCs [87, 88, 93, 94]. In the following experiments, it was tested whether mTORC1 is involved in the regulation of GSK3 in NPCs using chemical inhibitors. Primary E14.5 NPCs were differentiated with DMEM containing 2% FBS and treated with 20 nM rapamycin alone or with 5  $\mu$ M SB216763 for 3 days. The activities of SB216763 and rapamycin were confirmed by measuring the phosphorylation level of their downstream target proteins, p-GSK3 $\alpha$  (Tyr279), p-GSK3 $\beta$  (Tyr216) and p-P70S6K (Thr389), using Western blot analysis (Fig. IV-1A, lanes 2 and 3). Upon SB216763 and rapamycin treatment for 3 hr, tyrosine phosphorylation of both GSK3 isoforms and p-P70S6K levels were decreased, respectively. When cells were treated with both inhibitors, the level of the downstream targets of both inhibitors was decreased. These data showed that rapamycin did not influence the inhibitory effect of SB216763 or vice versa (Fig. IV-1A, lane 4). Upon 20 nM rapamycin treatment, the percentage of TuJ1-positive cells was reduced from 51% to 8%, but 5  $\mu$ M SB216763 completely reversed this effect (Fig. IV-9A). A similar observation was made in the C17.2 murine immortalized neural stem cell line using SB216763 and LiCl (another well-known GSK3 inhibitor) (data not shown). Again, the proportion of Nestin-positive cells increased from 55% to 73% after rapamycin treatment, and the extent of this increase was reduced by SB216763 co-treatment. The percentage of GFAP-positive cells was not affected by rapamycin or SB216763. These data suggest that GSK3 might inhibit neuronal differentiation as a downstream target of mTORC1.



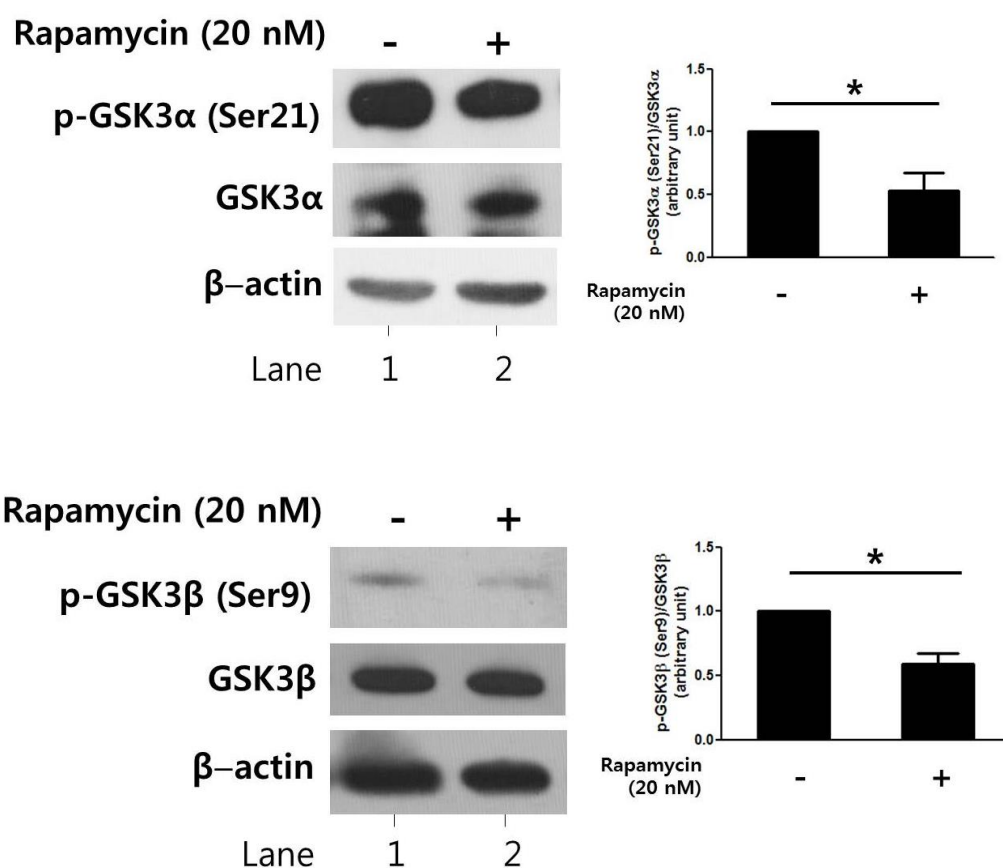


**Figure IV-8. GSK3 $\beta$  does not affect the migrating ability of NPCs.** Control or GSK3 $\beta$ -overexpressing NPCs were sorted, and their migrating ability was measured using transwell migration assay. The numbers of cells that migrated to the bottom of the membrane were counted. Scale bar, 100  $\mu$ m. \*\*\* $P < 0.001$ ; C, control.



It was hypothesized that GSK3 $\alpha$  and GSK3 $\beta$  might be differentially regulated by mTORC1. Because SB216763 inhibits both GSK3 $\alpha$  and GSK3 $\beta$ , shRNAs targeting each isoform were employed to test the effect of mTORC1 on each isoform. Primary NPCs were transduced with retroviral vectors expressing shRNA against GSK3 $\alpha$  or GSK3 $\beta$  and induced to differentiate in the presence of 20 nM rapamycin for 2 days (Fig. IV-9B). Similar to the above results, rapamycin treatment reduced the number of immature neurons by approximately 65% in the control group (from 31.5% to 11.2%). The level of reduction was similar when GSK3 $\alpha$  shRNA-expressing cells were treated with rapamycin. However, this reduction was not observed in the GSK3 $\beta$  shRNA-expressing group. As expected, knock-down of the expression of GSK3 $\beta$  produced a proportion of TuJ1- positive cells that was greater than that of the control group (Fig. IV-9B, white bar), similar to the result shown in Fig. IV-3. The proportion of Nestin-positive cells was increased approximately 1.5-fold when control and GSK3 $\alpha$ -silenced cells were treated with rapamycin. When GSK3 $\beta$  expression was lowered by shRNA, this increase was significantly reduced, and only approximately 23% of the GSK3 $\beta$  shRNA-expressing cells remained as NPCs. Therefore, these data not only confirm that rapamycin inhibited neuronal differentiation by blocking the repression of GSK3 activity but also clearly demonstrate that GSK3 $\alpha$  and GSK3 $\beta$  were differentially affected by the upstream regulator mTORC1.

As mTORC1 is known to regulate GSK3 via phosphorylation of the inhibitory serine residue [85, 86], the effects of mTORC1 on serine phosphorylation of GSK3 $\alpha$  and GSK3 $\beta$  were investigated in NPCs. Primary NPCs were treated with 20 nM rapamycin for 1 day and proteins were prepared and analyzed by western blotting. The serine phosphorylation levels of both GSK3 $\alpha$  and GSK3 $\beta$  were decreased by 50.7% and 37.6% upon rapamycin treatment, respectively (Fig. IV-10). These data indicate that the inhibition of mTORC1 resulted in reduced serine phosphorylation of both GSK3 $\alpha$  and GSK3 $\beta$ , suggesting that other regulatory mechanisms might be involved in controlling the activity of GSK3 isoforms downstream of mTORC1.



**Figure IV-10. Serine phosphorylation of both GSK3α and GSK3β are reduced upon the inhibition of mTORC1.** Proteins from the primary NPCs induced to differentiate in the presence of rapamycin for 1 day were prepared and subjected to western blot using antibodies to p-GSK3α (Ser21) and p-GSK3β (Ser9). The band intensity was quantified by Image J software. DNA was labeled with Hoechst 33258. Scale bar, 50 μm. \*P < 0.05; \*\*\*P < 0.001; n.s., not significant; C, control.

### 3. Discussion

Prior to this study, the roles of the two isoforms of GSK3, GSK3 $\alpha$  and GSK3 $\beta$ , in the differentiation of NPCs were unclear. To identify the functions of each isoform in brain development, the expression levels of GSK3 $\alpha$  and GSK3 $\beta$  were manipulated separately in NPCs through the downregulation or overexpression of each isoform. Knock-down of GSK3 $\beta$  expression led to increased neurogenesis, while stable expression of GSK3 $\beta$  suppressed neurogenesis and helped to maintain NPC characteristics both *in vitro* and *in vivo*. By contrast, GSK3 $\alpha$  alone did not produce any noticeable effects. These data are in agreement with the results of experiments involving *Drosophila* that are defective in the *shaggy* gene, a GSK3 homologue, which exhibit bristle hyperplasia on the thorax [77].

These results contrast with the findings of a double KO study by Kim *et al.* [76] which showed that the loss of GSK3 induces hyperproliferation of NPCs and decreases neuronal differentiation. Although the reason for the discrepancy between these two studies is not clear, the germ-line deletion of GSK3 $\alpha$  might have caused indirect effects, as GSK3 is an important regulator during embryogenesis [95]. Both GSK3 $\alpha$  and GSK3 $\beta$  are known to be expressed from the two-cell stage embryo, and their activities are tightly controlled as the embryo develops [96]. Therefore, the germ-line manipulation of either form of GSK3 may have produced unexpected outcomes in the later stages of development, especially when the other form, which could have compensated for the loss, was deleted. In this study, I controlled the expression level of each isoform at a relatively late stage of embryonic development and demonstrated that GSK3 $\beta$  acts as a negative regulator during embryonic neurogenesis.

The results described in this chapter suggest that the two isoforms can have two different consequences in the context of neuronal differentiation. Considering the different life spans of GSK3 $\alpha$  and GSK3 $\beta$  single KO mice [76, 97, 98], it is possible that GSK3 $\alpha$  may not be able to complement all the defects caused by the absence of GSK3 $\beta$ . This possibility could indicate that the two isoforms target different populations of cellular proteins. GSK3 has been shown to play roles in many different

signaling pathways that are involved in neural cell proliferation and differentiation including Wnt, Shh, Notch, and c-Myc [99-103]; however, all of the studies that demonstrated these effects were performed only with GSK3 $\beta$ . Thus, GSK3 $\alpha$  might not target the same proteins as GSK3 $\beta$ , and even if it does, the regulatory activities of each isoform may be manipulated differently.

The exact mechanisms by which GSK3 $\alpha$  and GSK3 $\beta$  are regulated require further investigation. In this study, rapamycin failed to suppress neuronal differentiation in GSK3 $\beta$ -, but not GSK3 $\alpha$ -, knock-down cells, indicating that mTORC1 might not affect the activity of GSK3 $\alpha$ . Other investigators have indicated that the functions of GSK3 $\alpha$  and GSK3 $\beta$  can be regulated independently. For example, dephosphorylation of the N-terminal regions of the two isoforms has been shown to be regulated by different phosphatases, PP2A for GSK3 $\alpha$  and PP1 for GSK3 $\beta$ . It was also reported that GSK3 $\alpha$  was preferentially localized in the nucleus compared to GSK3 $\beta$ , which might allow GSK3 $\alpha$  to be more competent in phosphorylating its target protein [104]. Whatever the case, these data unequivocally demonstrate that GSK3 $\beta$ , but not GSK3 $\alpha$ , is a key mediator of NPC differentiation.

A recent publication showed that the selective silencing of either GSK3 $\alpha$  or GSK3 $\beta$  leads to different consequences in mouse models of Alzheimer's disease [105]. GSK3 $\alpha$ -, but not GSK3 $\beta$ -, silenced amyloid precursor protein (APP) transgenic mice exhibited reduced levels of amyloid  $\beta$  formation in the adult hippocampus, which indicates the characteristic roles of GSK3 $\alpha$  and GSK3 $\beta$  in neurodegenerative disease. GSK3 is known to be a critical protein involved in numerous central nervous system diseases including bipolar disorder, Alzheimer's disease, and schizophrenia [106-108]. Thus, the study of GSK3 isoforms is critical not only for the understanding of these serious human disorders but also for the development of new and effective therapeutic strategies.

## **CHAPTER V**

# **Effects of IFN- $\gamma$ on Embryonic Neurogenesis**

# 1. Background

IFN- $\gamma$  is one of the inflammatory cytokines expressed in the fetal brain following an inflammatory stimulus [109]. It can be expressed from E7 in mice and week 21 of pregnancy in humans [110, 111]. The binding of IFN- $\gamma$  to its receptor results in the phosphorylation of JAK1 and JAK2, and the subsequent phosphorylation of STAT1. Phosphorylated STAT1 then forms a homodimer and translocates to the nucleus [112]. STAT1, acting as a transcription factor, can positively or negatively regulate downstream target genes, depending on the presence of other cofactors [113].

The effects of IFN- $\gamma$  on neurogenesis have been investigated *in vitro* using recombinant IFN- $\gamma$  in various types of cells. However, depending on the cell type and the concentration of IFN- $\gamma$ , different results have been reported. In experiments involving murine adult neural stem cells, a human neuroblastoma cell line, a neonatal NPC line (C17.2), and E15-E16 neurons, treatment with IFN- $\gamma$  resulted in increased neurogenesis [114-117]. However, in an experiment involving cells from the subventricular zone of postnatal day 2 mouse brains, IFN- $\gamma$  had the opposite effect [118]. Furthermore, it was also reported that a high level of IFN- $\gamma$  resulted in irregular cell types double-positive for GFAP and TuJ1 when E14 neural stem/precursor cells were used [119]. Not only are the effects of IFN- $\gamma$  on neurogenesis controversial, but the downstream mechanism of this cytokine is barely elucidated.

In research described in this chapter, the role of IFN- $\gamma$  in embryonic neurogenesis was investigated both *in vitro* and *in vivo*, using primary E14.5 NPCs and E9.5 embryonic brains, respectively. It was found that IFN- $\gamma$  effectively inhibits neurogenesis, and this inhibition was dependent on the JAK/STAT1 signaling pathway in primary NPCs. An *in vivo* study using E14.5 embryonic brains also showed a similar result. Interestingly, IFN- $\gamma$  treatment specifically decreased the RNA level of *Neurogenin 2* (*Neurog2*) among other proneural genes.

# 2. Results

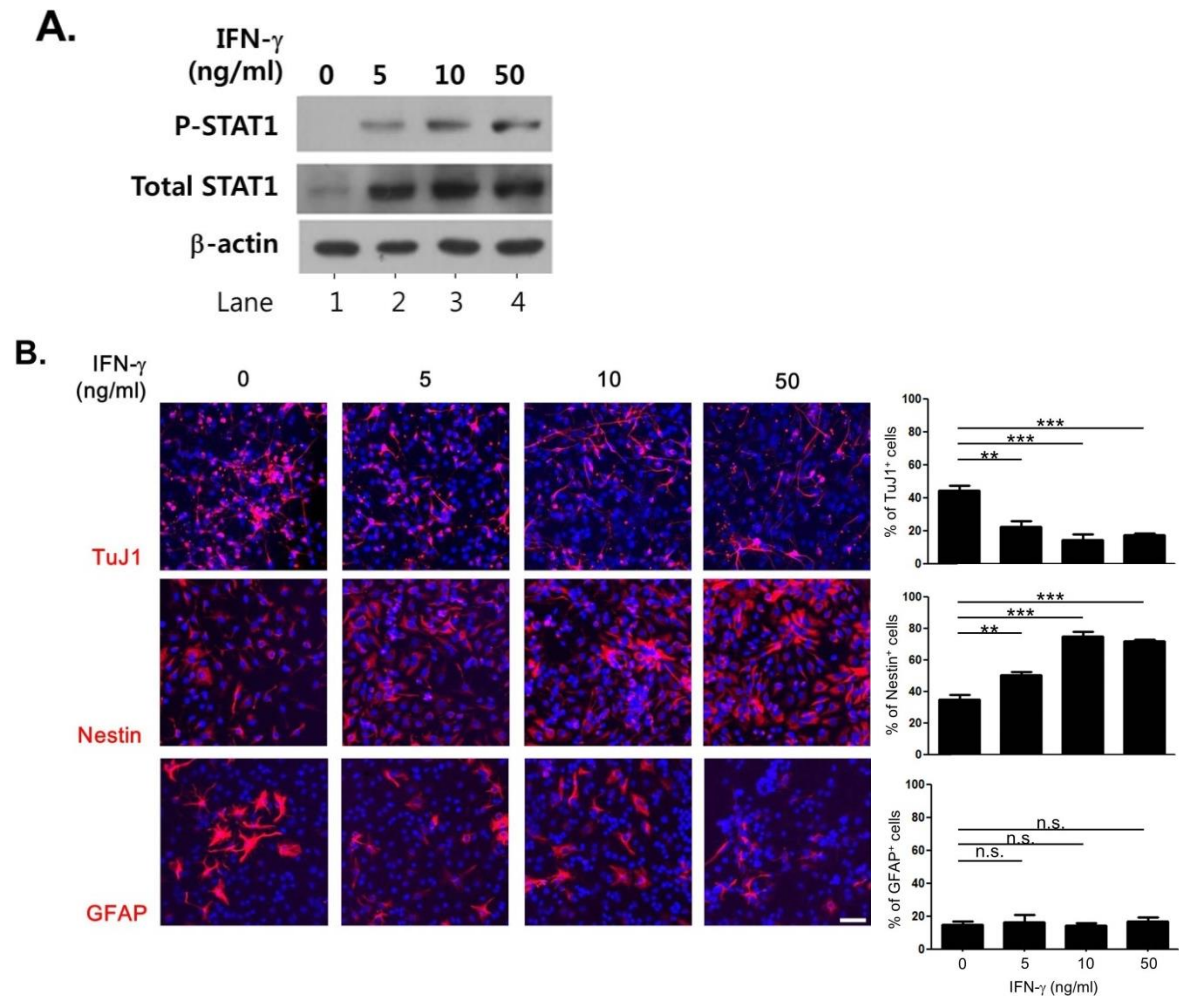


## 2.1. Effects of IFN- $\gamma$ on the neuronal differentiation of NPCs

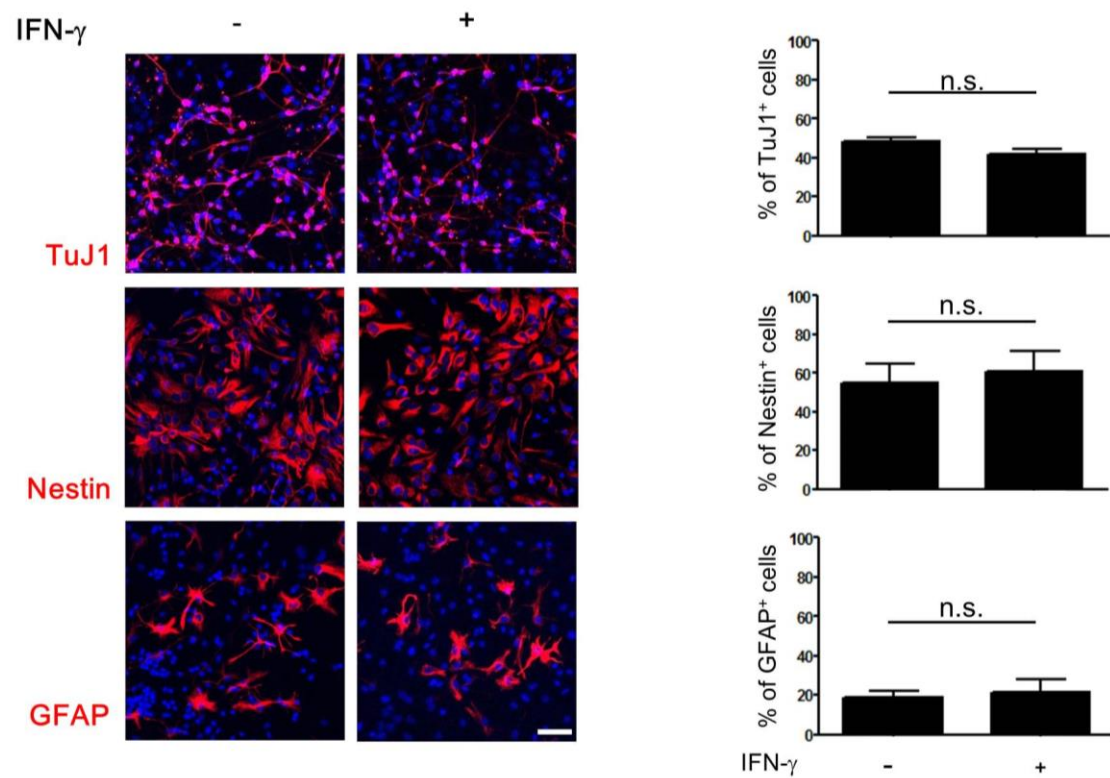
Overexpression of IFN- $\gamma$  in NPCs via retroviral transduction process could be problematic as the transduction efficiency and amount of IFN- $\gamma$  released by each experimental group may vary every time. Thus, to investigate the effects of IFN- $\gamma$  on neuronal differentiation in a more controllable manner, recombinant IFN- $\gamma$  was used. Primary NPCs were prepared from E14.5 mouse embryos and cultured in NPC proliferation medium. After 2 days, NPCs were differentiated with DMEM containing 2% FBS in the presence of 5-50 ng/mL IFN- $\gamma$  for another 2 days. To test the functionality of IFN- $\gamma$  used in this study, the status and level of STAT1 were analyzed. As expected, the amount of Tyr701-phosphorylated STAT1 and the amount of total STAT1 increased as the concentration of IFN- $\gamma$  increased, indicating that the recombinant IFN- $\gamma$  is active (Fig.V-1A). Differentiated cells were stained for specific markers of immature neurons (TuJ1), NPCs (Nestin), and astrocytes (GFAP). 45 $\pm$ 5.2% of total cells were TuJ1-positive immature neurons, while 35 $\pm$ 5.3% of them were Nestin-positive NPCs. Only a small proportion of cells (15 $\pm$ 1.2%) were GFAP-positive astrocytes. Upon treatment with 50 ng/mL IFN- $\gamma$ , the percentage of TuJ1-positive cells was decreased to 17 $\pm$ 2.0%, while the percentage of Nestin-positive cells was increased to 72 $\pm$ 1.2%. There was no significant difference in the percentage of GFAP-positive astrocytes (Fig. V-1B). Similar to the results from Chapter III, these results strongly suggest an inhibitory role of IFN- $\gamma$  in the neuronal differentiation of NPCs.

To test whether IFN- $\gamma$  has an effect on NPCs in a proliferative status, isolated NPCs were grown in NPC proliferation medium with 50 ng/mL IFN- $\gamma$  for 2 days and differentiated in the absence of IFN- $\gamma$ . There were no statistically significant differences in the percentages of different cell types between the control and IFN- $\gamma$ -treated cells (Fig. V-2). This indicates that IFN- $\gamma$  may have effects only on NPCs that have started differentiation and not on actively proliferating NPCs.

To investigate whether the decreased number of TuJ1-positive cells was due to increased cell



**Figure V-1. IFN- $\gamma$  decreases the neuronal differentiation of primary NPCs.** (A) NPCs were differentiated with various concentrations of IFN- $\gamma$ . After 3 hr, whole cell lysates were prepared and the level of phosphorylated STAT1 was observed by western blotting using antibodies to P-STAT1 (Tyr701), total STAT1 and  $\beta$ -actin. (B) Primary NPCs were induced to differentiate in the presence of different concentrations of IFN- $\gamma$  for 2 days and then immunostained for different cell markers (TuJ1, Nestin, and GFAP). Representative images of the staining and the percentages of each cell type are shown.



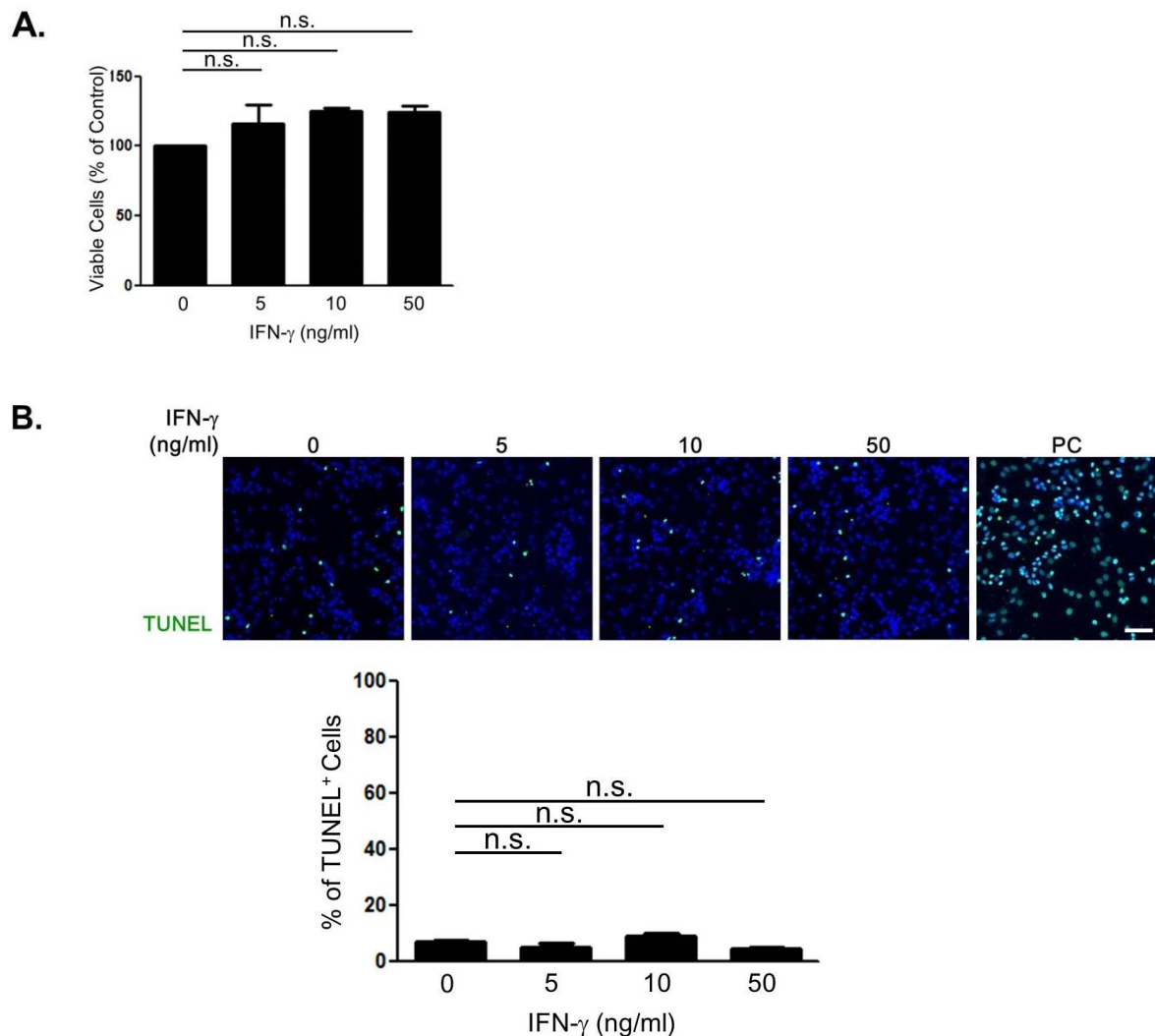
**Figure V-2. IFN- $\gamma$  has no effect on proliferating NPCs.** Primary NPCs were grown in proliferation medium in the presence of 50 ng/mL IFN- $\gamma$  for 2 days and induced to differentiate in the absence of IFN- $\gamma$ . After 2 days, immunostaining was done for TuJ1, Nestin, and GFAP. Representative images of the staining and the percentages of each cell type are shown.

death, NPCs were differentiated with 5-50 ng/mL IFN- $\gamma$  for 2 days and subjected to MTT and TUNEL assays. As shown in Fig.V-3A, no statistically significant difference was found in the number of viable cells between the control and IFN- $\gamma$ -treated groups. Moreover, similar numbers of the TUNEL-positive cells were observed in the control and IFN- $\gamma$ -treated groups (Fig. V-3B). These data demonstrate that IFN- $\gamma$  does not have cellular toxicity and does not have an effect on apoptotic cell death.

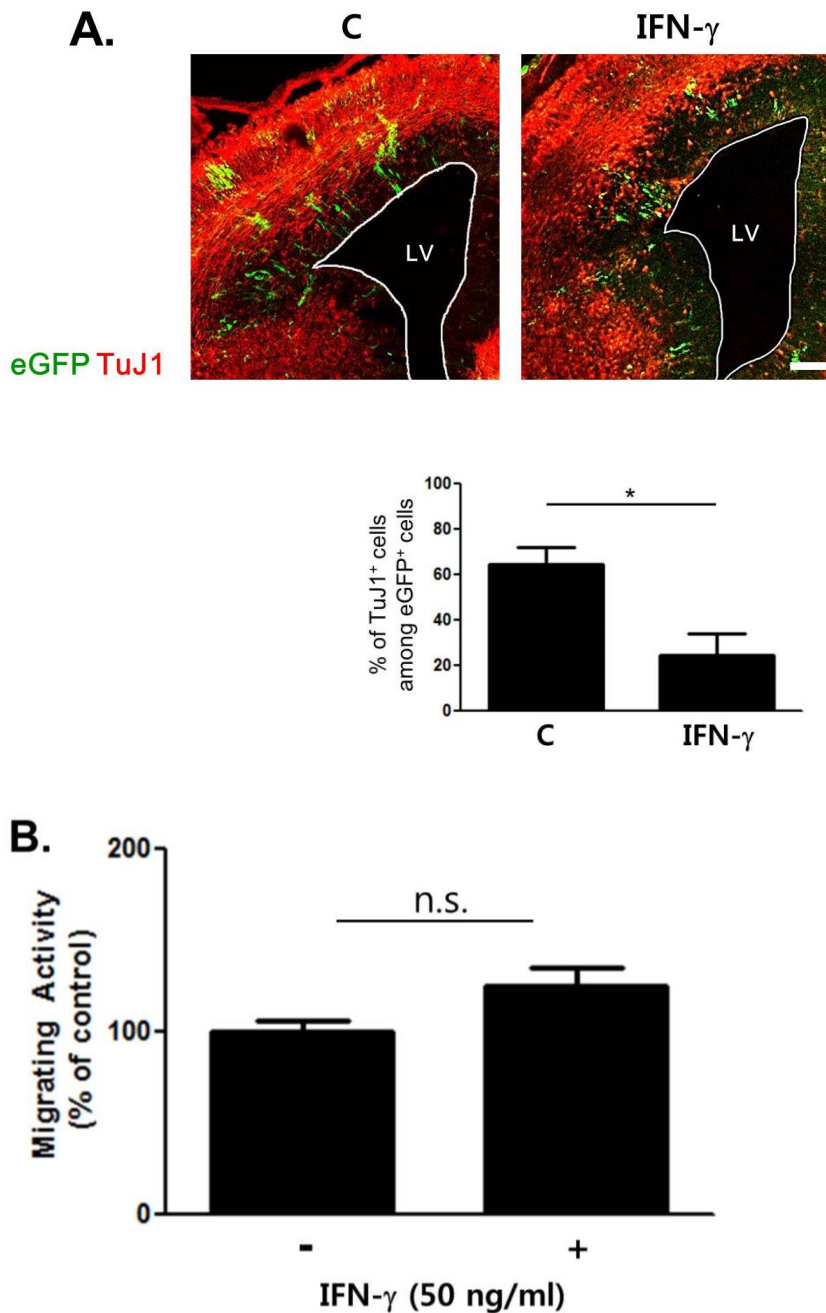
## **2.2. Effects of IFN- $\gamma$ on the neuronal differentiation of NPCs *in vivo***

To confirm the inhibitory effect of IFN- $\gamma$  on neuronal differentiation *in vivo*, a retroviral vector, expressing IFN- $\gamma$  and eGFP from a bicistronic message, was injected into the ventricles of E9.5 embryonic brains using the UIGD technique [120]. As described in the previous chapter, when NPCs in the VZ are transduced with such retroviral vectors, the neurons produced from the NPCs are supposed to migrate away from the VZ during neurogenesis. The brains were analyzed at E14.5, when neurogenesis reaches its peak level [121]. When embryonic brains were injected with a control vector expressing only eGFP, 64 $\pm$ 13% of eGFP-positive cells were localized in the TuJ1-stained region. By contrast, when the vector expressing both IFN- $\gamma$  and eGFP was delivered to embryonic brains, the transduced cells were predominantly found in the VZ (Fig.V-4A). Only approximately 24% of cells were positive for TuJ1, which was significantly lower than that of the control ( $P=0.0287$ ). These data suggest that IFN- $\gamma$  indeed suppresses neuronal differentiation during brain development.

To determine whether the retention of NPCs in the VZ in the IFN- $\gamma$  group resulted from the inhibition of cell migration, the effects of IFN- $\gamma$  on the migration of NPCs were also tested. Primary NPCs were allowed to form neurospheres and were allowed to differentiate with or without IFN- $\gamma$  for 1 day. To determine the migrating ability of NPCs, the distance from the edge of the neurosphere to the furthest outgrowing cell was measured and normalized to the diameter of the neurosphere (Fig. V-4B). There was no statistically significant difference between the control and the group treated with IFN- $\gamma$ , suggesting that IFN- $\gamma$  treatment does not affect the migrating ability of NPCs.



**Figure V-3. IFN- $\gamma$  does not affect the viability and the apoptosis of primary NPCs.** (A) Primary NPCs were differentiated with various concentrations of IFN- $\gamma$  for 2 days and subjected to the MTT assay. The percentages of viable cells compared with the control are shown. (B) Primary NPCs were differentiated with different concentrations of IFN- $\gamma$  for 2 days, and then the TUNEL assay was performed. DNase-treated cells were used as a positive control (PC). Representative images of the TUNEL staining and the percentage of TUNEL-positive cells are shown. DNA was labeled with Hoechst 33258 (blue). Scale bar, 50  $\mu$ m. The statistical significance of differences between two groups was analyzed using the unpaired *t* test. Other differences in values were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. \*\**P* < 0.01; \*\*\**P* < 0.001; n.s., not significant.



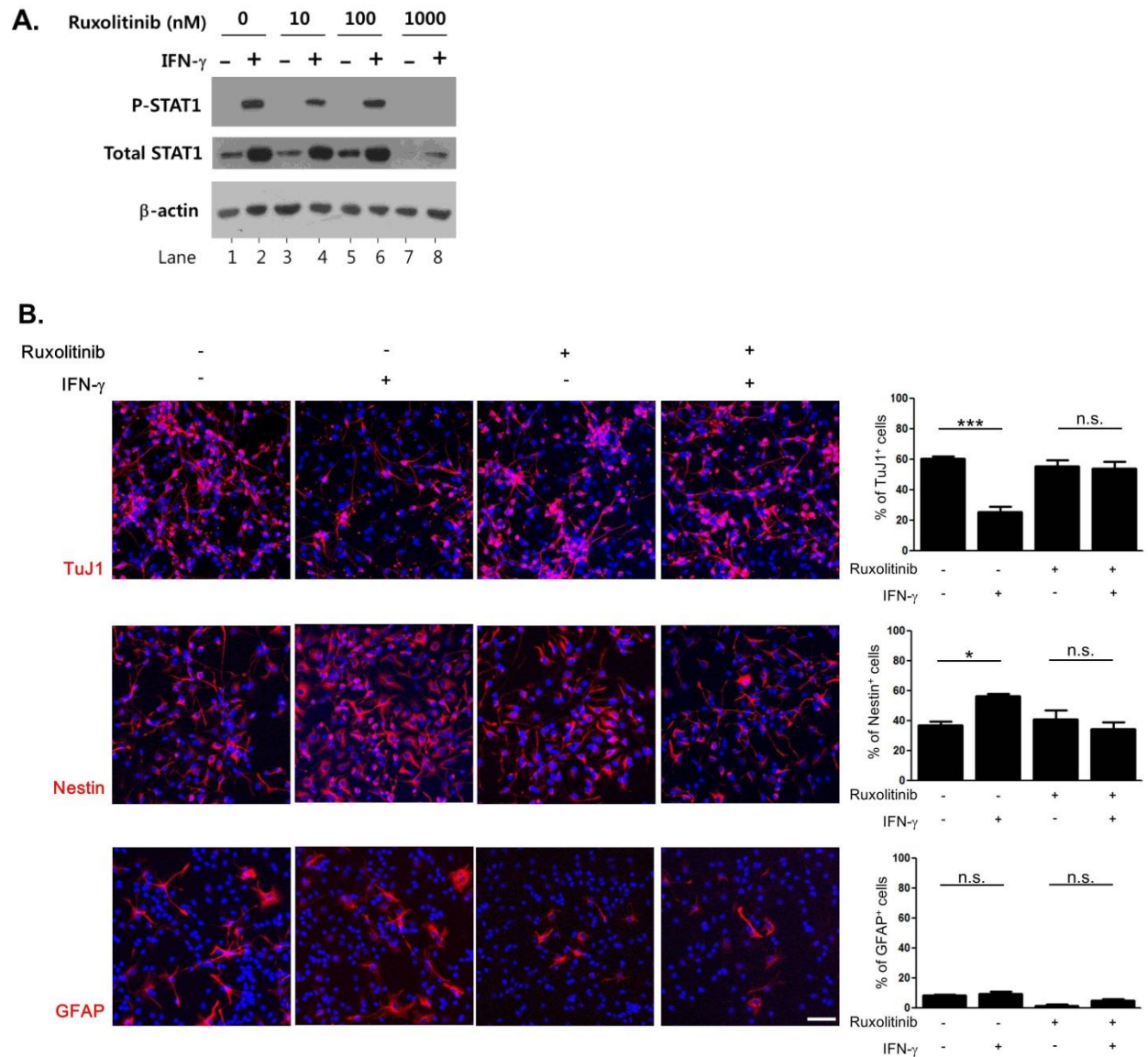
**Figure V-4. IFN- $\gamma$  inhibits neuronal differentiation *in vivo*.** (A) Retroviral vectors expressing IFN- $\gamma$  and eGFP were injected into E9.5 embryonic brains ( $n = 3/\text{group}$ ) and then the brains were immunostained with antibodies to eGFP and TuJ1 at E14.5. Representative images of sections and the percentages of TuJ1-positive neurons among GFP-positive cells are shown. (B) Primary neurospheres were differentiated with 50 ng/mL IFN- $\gamma$  for 1 day. Migrating activity was calculated by measuring the distance from the edge of the neurosphere to the furthest outgrowing cell and the diameter of the neurospheres. Scale bar, 100  $\mu\text{m}$ . The statistical significance of differences was analyzed using the unpaired  $t$  test. \* $P < 0.05$ . LV, lateral ventricle; C, control.

### **2.3. Involvement of the JAK/STAT1 pathway in the IFN- $\gamma$ -mediated inhibition of neurogenesis**

To understand the downstream mechanism of the inhibitory role of IFN- $\gamma$  in embryonic neurogenesis, the involvement of the JAK/STAT1 pathway was analyzed as it is the main signaling route activated by IFN- $\gamma$ . Primary E14.5 NPCs were differentiated with DMEM containing 2% FBS and treated with IFN- $\gamma$  alone or in combination with 1  $\mu$ M JAK1/2 inhibitor (Ruxolitinib) for 2 days. The activity of Ruxolitinib was confirmed by examining the phosphorylation level of STAT1 by western blotting. Upon IFN- $\gamma$  treatment, the STAT1 tyrosine residue was phosphorylated as expected, but phosphorylation was totally inhibited when 1  $\mu$ M Ruxolitinib was added. The level of total STAT1 was also decreased upon 1  $\mu$ M Ruxolitinib treatment. Since Ruxolitinib is a JAK1/2 inhibitor, proteins other than STAT1 that are activated by JAK1/2 could have affected the level of total STAT1 (Fig.V-5A). The proportion of TuJ1-positive cells was reduced from  $60\pm 3.1\%$  to  $26\pm 5.6\%$  upon IFN- $\gamma$  treatment and was restored to the control level when Ruxolitinib was added concurrently. Conversely, the proportion of Nestin-positive cells increased from  $36\pm 4.8\%$  to  $56\pm 2.9\%$  after IFN- $\gamma$  treatment but remained similar to the control level after treatment with both IFN- $\gamma$  and Ruxolitinib ( $41\pm 11\%$  and  $34\pm 8.1\%$ , respectively). There was no significant difference in the number of GFAP-positive astrocytes between the control and IFN- $\gamma$ -treated groups (Fig.V-5B).

As JAK1/2 is involved in the activation of not only STAT1 but also other signaling pathways such as STAT3 and MAPK pathways [122-124], shRNA targeting STAT1 was employed to confirm the role of STAT1 and rule out the effects of other factors on neurogenesis. Retroviral vectors expressing three different shRNA sequences against STAT1, together with HA-tagged DsRed, were constructed. To determine the knock-down efficiency, NIH3T3 cells were transduced with retroviral vectors expressing control or STAT1 shRNA. Two days later, the level of STAT1 was measured by western blotting. STAT1 shRNA sequence #2 showed the strongest inhibition of STAT1 expression (approximately 67.2%) (Fig. V-6A), and therefore was chosen for subsequent experiments. NPCs were transduced with the retroviral vector expressing STAT1 shRNA and, 2 days later, induced to





**Figure V-5. Inhibition of the JAK/STAT1 pathway abrogated the IFN- $\gamma$ -mediated inhibition of neurogenesis. (A)** NPCs were induced to differentiate in the presence of different concentrations of Ruxolitinib for 12 hr. Proteins were prepared and subjected to Western blot using antibodies to P-STAT1 (Tyr701), total STAT1 and  $\beta$ -actin. **(B)** NPCs were induced to differentiate in the presence of 50 ng/mL IFN- $\gamma$  with or without 1  $\mu$ M Ruxolitinib for 2 days and then immunostained for TuJ1, Nestin, and GFAP. Representative images of the staining and the percentages of each cell type are shown.

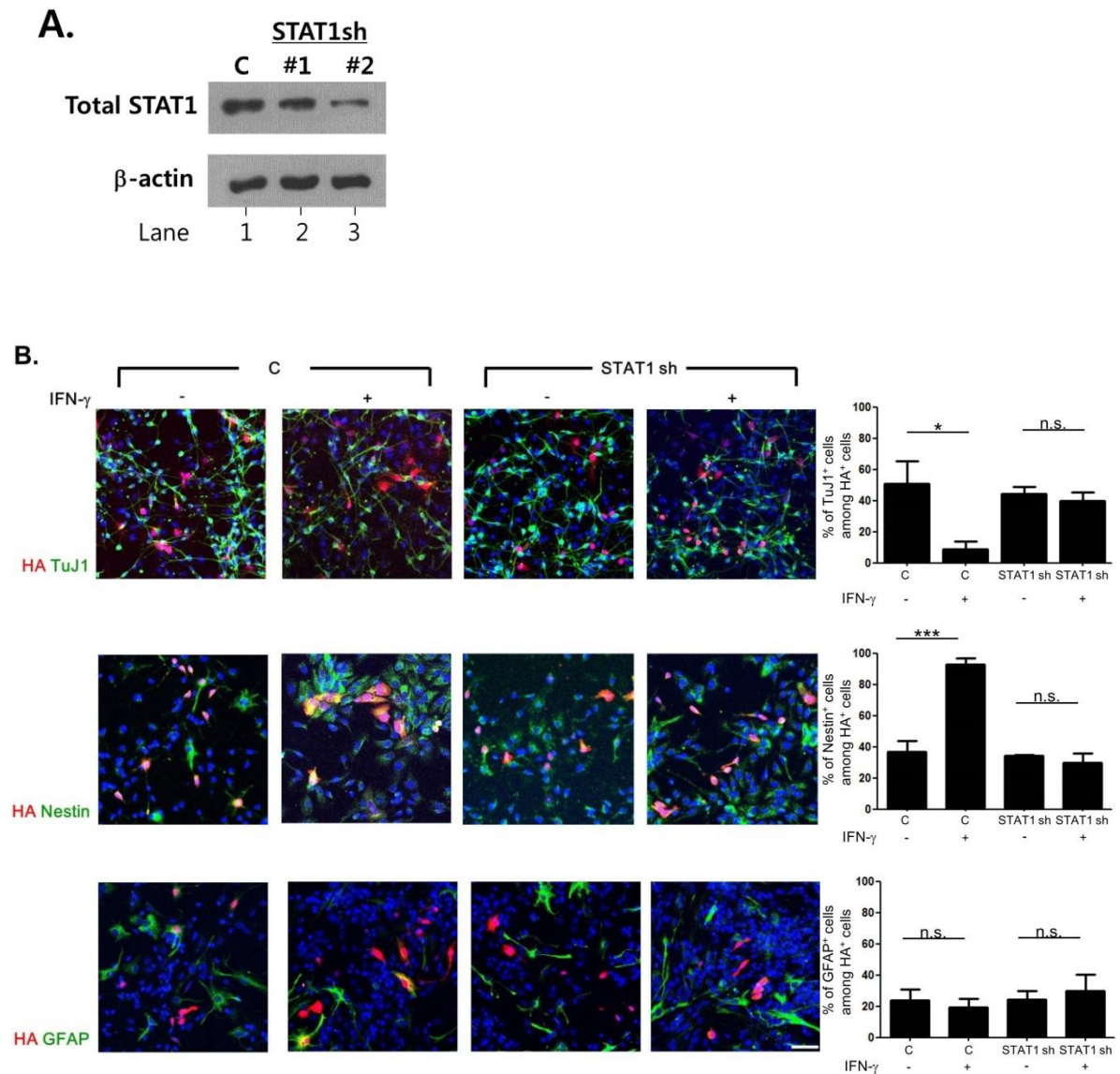


differentiate in the presence of IFN- $\gamma$  for 2 days. The percentages of HA-positive cells expressing different cell markers were calculated. Among the NPCs transduced with the control vector, the percentage of TuJ1-positive cells was reduced from  $51\pm 25\%$  to  $10\pm 8.4\%$  upon IFN- $\gamma$  treatment. When cells were transduced with the vector expressing STAT1 shRNA, however, there was no statistically significant difference between the control and IFN- $\gamma$ -treated groups ( $44\pm 7.9\%$  and  $40\pm 10\%$ , respectively). The percentage of Nestin-positive cells was increased approximately 2.5-fold upon IFN- $\gamma$  treatment, while such an increase was not observed in cells expressing STAT1 shRNA. Neither IFN- $\gamma$  treatment nor the knock-down of STAT1 expression affected the number of GFAP-positive cells (Fig. V-6B). These results clearly show that the inhibition of neurogenesis by IFN- $\gamma$  is dependent on the JAK/STAT1 signaling pathway.

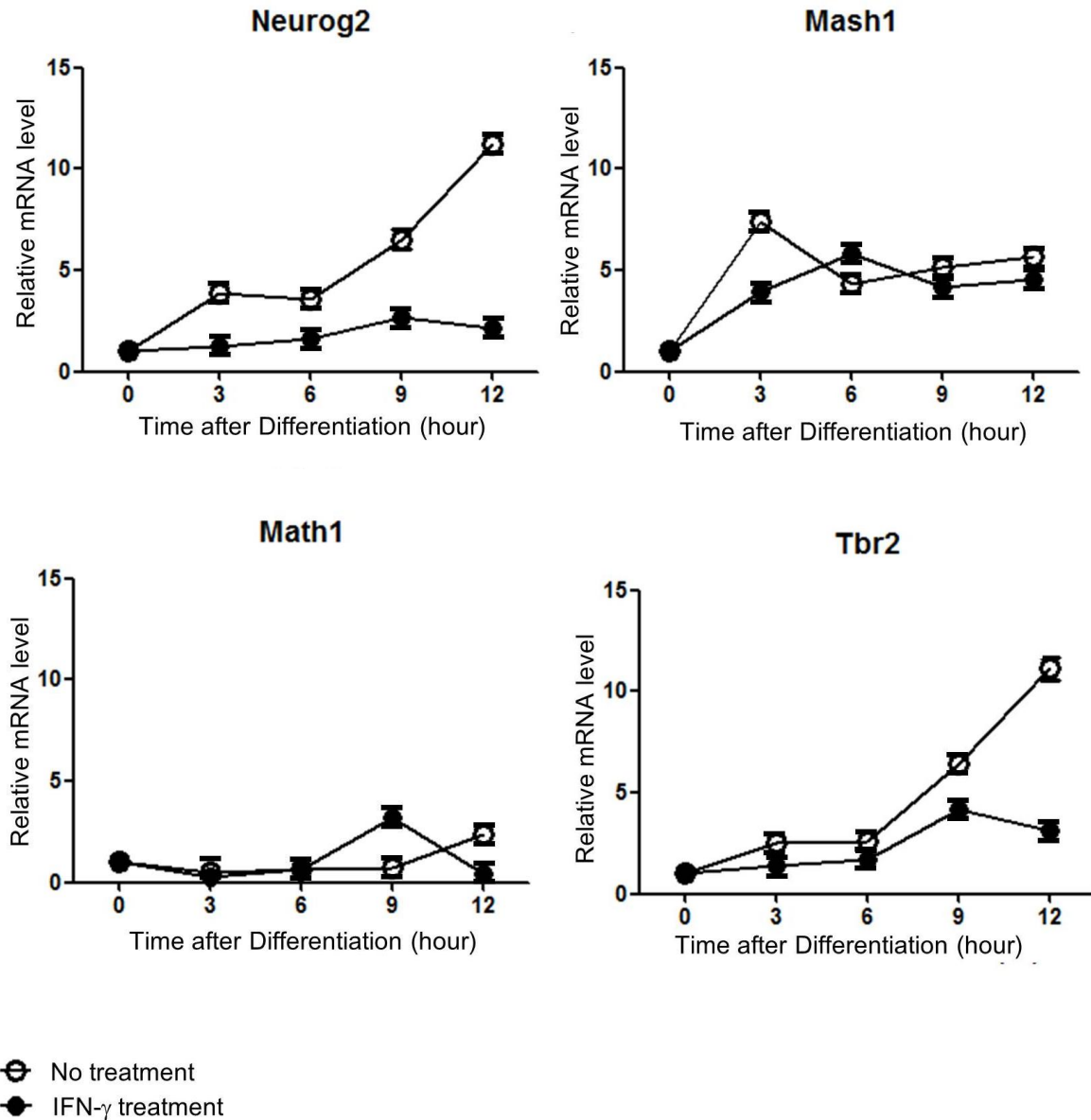
## 2.4. Effects of IFN- $\gamma$ on the expression of *Neurogenin 2*

It was tested whether IFN- $\gamma$  had an effect on the expression of the bHLH proneural genes as they are key players in the neuronal differentiation of NPCs [125]. Primary NPCs were treated with IFN- $\gamma$  and differentiated with DMEM containing 2% FBS. Total RNA was collected every 3 hr up to 12 hr, and the RNA levels of *Neurog2*, *Mash1* and *Math1* were analyzed by qRT-PCR. While the expression levels of *Mash1* and *Math1* were similar between the control and IFN- $\gamma$ -treated groups, there was a remarkable change in the case of *Neurog2*. The RNA level of *Neurog2* was induced more than 10-fold upon differentiation, while this induction was almost completely inhibited in the IFN- $\gamma$ -treated group (Fig. V-7). To determine whether an increased level of *Neurog2* expression can generate actual biological effects, the expression level of *Tbr2*, one of the downstream targets of *Neurog2*, was measured [126]. The RNA level of *Tbr2* was also increased in a time-dependent manner upon differentiation, while its upregulation was reduced upon treatment with IFN- $\gamma$ , correlating with the level of *Neurog2* expression.

To investigate whether *Neurog2* is indeed the key downstream target of IFN- $\gamma$ , NPCs were transduced with a retroviral vector expressing *Neurog2* and eGFP from a bicistronic message for 2



**Figure V-6. IFN- $\gamma$ -mediated inhibition of neurogenesis depends on the JAK/STAT1 pathway.** (A) NIH3T3 cells were transduced with the same titer of retroviral vectors expressing control or STAT1 shRNA. After 2 days, proteins were prepared and subjected to Western blot using antibodies to total STAT1 and  $\beta$ -actin. (B) Primary NPCs were transduced with retroviral vectors expressing control or STAT1 shRNA together with HA-tagged DsRed. After 2 days, cells were differentiated with or without IFN- $\gamma$  and then co-immunostained for HA and different cell markers (TuJ1, Nestin, and GFAP). Representative images of the staining and the percentages of each cell type among HA-positive cells are shown. DNA was labeled with Hoechst 33258 (blue). Scale bar, 50  $\mu$ m. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. \* $P < 0.05$ ; \*\*\* $P < 0.001$ ; n.s., not significant. C, control.



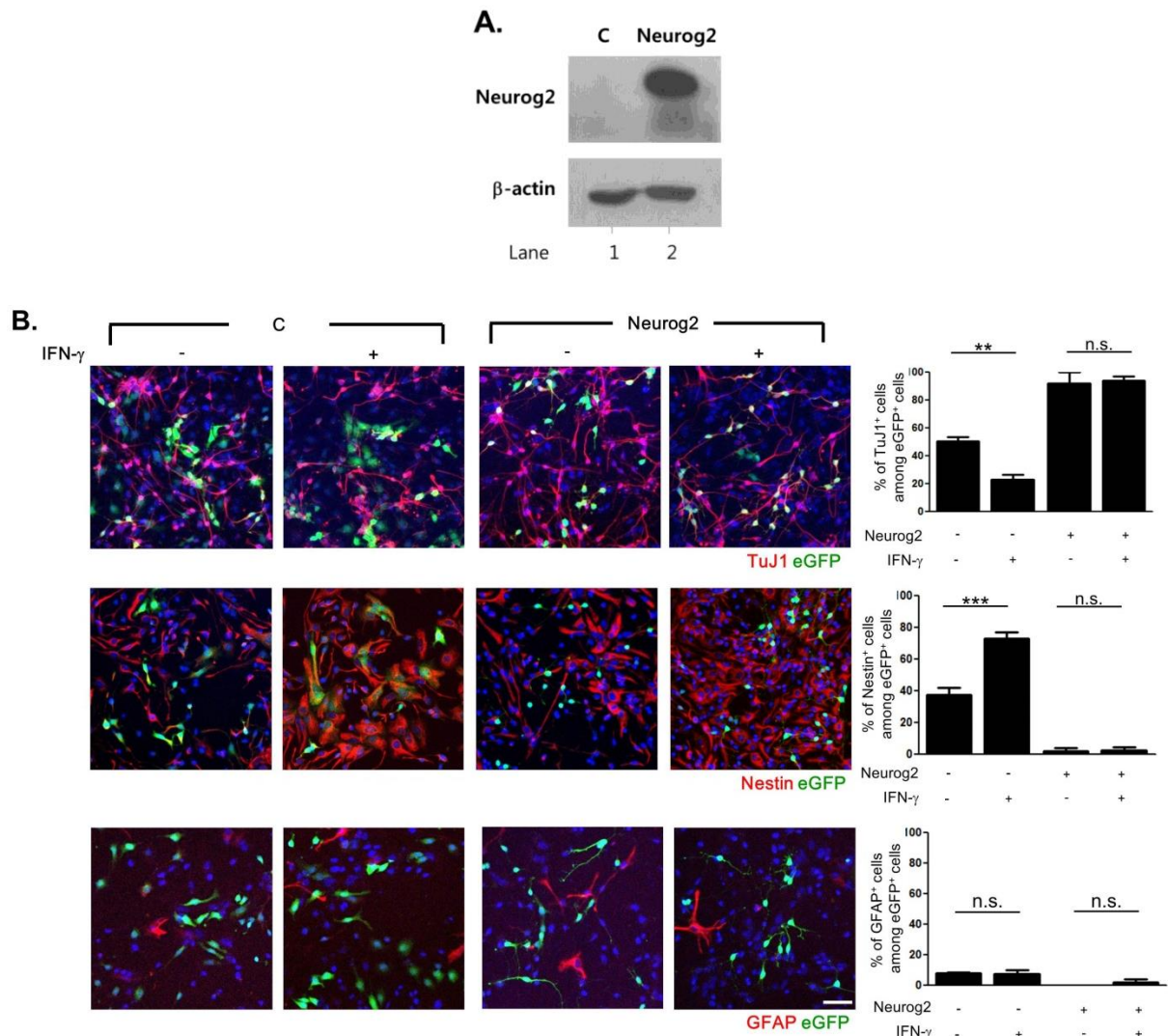
**Figure V-7. IFN- $\gamma$  negatively regulates the expression of *Neurog2* mRNA.** Total RNA was isolated from differentiated NPCs treated with or without 50 ng/mL IFN- $\gamma$  every 3 hr for 12 hr. The levels of *Neurog2*, *Math1*, *Mash1*, and *Tbr2* mRNA were measured by qRT-PCR. Differences between the values of two groups were analyzed by two-way ANOVA (Source of Variation = IFN- $\gamma$  treatment). P values for *Neurog2*, *Math1*, *Mash1*, and *Tbr2* are <0.0001, 0.3012, 0.3357, and 0.0001, respectively.

days and then differentiated with or without 50 ng/mL IFN- $\gamma$  for another 2 days. The expression of exogenous *Neurog2* was confirmed in NIH3T3 cells by western blotting (Fig. V-8A). IFN- $\gamma$  treatment did not affect the level of eGFP expression when NPCs were transduced with a control vector expressing eGFP only, suggesting that IFN- $\gamma$  does not have an influence on the function of viral long terminal repeats (data not shown). As expected, among the cells transduced with the control vector, IFN- $\gamma$  treatment reduced the number of TuJ1-positive cells by nearly half while increasing the number of Nestin-positive cells more than 2-fold. However, almost all cells overexpressing *Neurog2* had differentiated to TuJ1-positive neurons, and IFN- $\gamma$  treatment showed no inhibitory effect ( $92\pm 14\%$  and  $94\pm 5.2\%$ , respectively). There was no statistically significant difference in the percentage of GFAP-positive cells between the control and IFN- $\gamma$ -treated groups (Fig. V-8B). These results reveal that the inhibition of *Neurog2* expression is necessary for the IFN- $\gamma$ -mediated inhibition of neurogenesis.

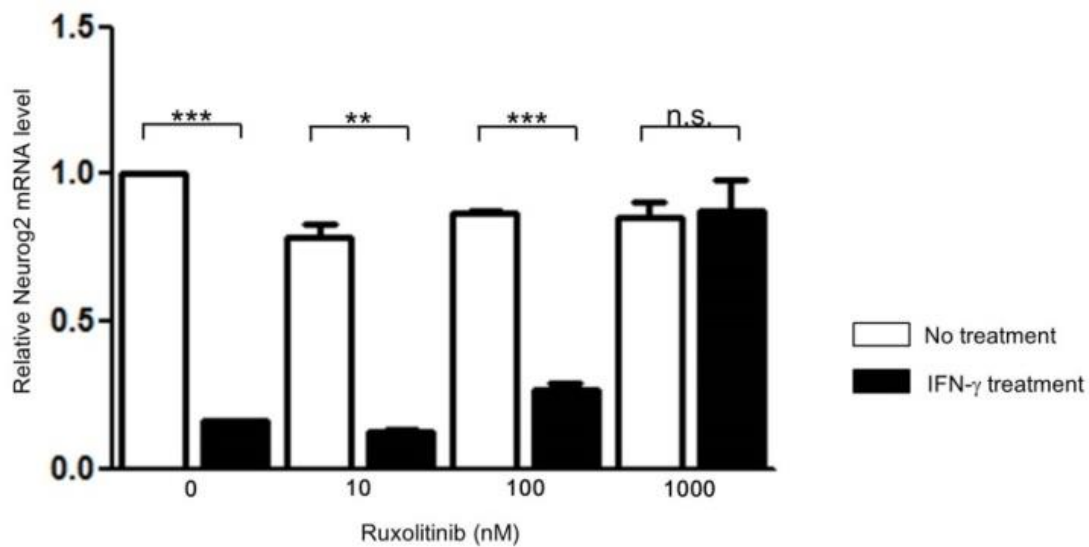
Since the effects of IFN- $\gamma$  on neurogenesis depend on the JAK/STAT1 pathway, it was tested whether inhibition of the JAK/STAT1 pathway would affect the IFN- $\gamma$ -mediated downregulation of *Neurog2* expression. NPCs were treated with IFN- $\gamma$  in the presence of 10 nM to 1  $\mu$ M Ruxolitinib and differentiated for 12 hr. Total RNA was isolated, and the level of *Neurog2* expression was determined by qRT-PCR (Fig. V-9). IFN- $\gamma$  treatment reduced the expression of *Neurog2* by approximately 84%, but the expression level was restored to that of the control at 1  $\mu$ M Ruxolitinib. Taken together, these data indicate that IFN- $\gamma$  inhibits neurogenesis by inhibiting *Neurog2* expression through the JAK/STAT1 pathway.

### 3. Discussion

The role of IFN- $\gamma$  in neurogenesis has been controversial. The results reported in this chapter demonstrate that IFN- $\gamma$  negatively regulates neuronal differentiation both *in vitro* and *in vivo*. However, these data are in contrast with the results of some previous studies. For example, a study involving the C17.2 cell line has showed that IFN- $\gamma$  induces neuronal differentiation by activating the JNK pathway [116]. In the study of Walter *et al.*, treatment of proliferating E14 neural stem/precursor



**Figure V-8. Overexpression of *Neurog2* abrogated the IFN- $\gamma$ -mediated negative regulation of neurogenesis.** (A) NIH3T3 cells were transduced with a retroviral vector expressing *Neurog2*. After 2 days, cell lysates were prepared and subjected to Western blot using antibodies to *Neurog2* and  $\beta$ -actin. (B) Primary NPCs were transduced with a retroviral vector expressing *Neurog2* and eGFP for 2 days. Cells were then differentiated with or without IFN- $\gamma$  for another 2 days and subjected to co-immunostaining for eGFP and TuJ1, Nestin, or GFAP. Representative images and the percentage of each cell type among the eGFP-positive cells are shown. DNA was labeled with Hoechst 33258 (blue). Scale bar, 50  $\mu$ m. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; n.s., not significant.



**Figure V-9. IFN- $\gamma$ -mediated downregulation of *Neurog2* expression is dependent on the JAK/STAT1 pathway.** Primary NPCs were differentiated in the presence of different concentrations of Ruxolitinib with or without 50 ng/mL IFN- $\gamma$ . After 12 hr, RNA was isolated and the level of *Neurog2* mRNA was measured by qRT-PCR. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; n.s., not significant.

cells with 1,000 U/mL IFN- $\gamma$  (equivalent to 1 mg/mL) resulted in atypical gene expression and cell functions through regulation of the Shh pathway [127]. None of the above observations were made in this study. The different findings obtained in these studies may be explained by the various types of cells and the different concentrations of IFN- $\gamma$  used, or they may be due to differences in other experimental conditions such as the time of NPC isolation and the availability of cofactors involved in the JAK/STAT1 pathway.

Many downstream targets of IFN- $\gamma$  are known, but they have not been extensively studied in the context of embryonic neuronal differentiation. According to the results obtained from this chapter, one of the final target genes of IFN- $\gamma$  appears to be *Neurog2*. *Neurog2* is an essential factor during the initiation of the neuronal differentiation of NPCs and it is sufficient to generate neurons from mouse embryonic stem cells [128-131]. Thus, *Neurog2* is a key factor in the determination of neural cell types and its expression pattern is tightly regulated, but only a few factors are known to regulate its expression [132-134]. In the present study, IFN- $\gamma$  specifically suppressed the RNA level of *Neurog2* among many proneural genes. IFN- $\gamma$  treatment inhibited the upregulation of *Neurog2* expression in NPCs throughout the differentiation period, and the overexpression of *Neurog2* completely abrogated the inhibitory effect of IFN- $\gamma$  on neurogenesis. Because STAT1 can regulate gene expression in various ways, there are many possible mechanisms by which it might control the level of *Neurog2* mRNA. Further investigations are required to unravel the precise mechanism of how the JAK/STAT1 pathway controls the expression level of *Neurog2* mRNA.

Several recent studies have suggested that the cause of neurological disorders in infants that survive congenital infections is the inflammatory response, rather than the pathogen itself [69]. Lipopolysaccharide or polyinosinic:polycytidylic acid (synthetic double-stranded RNA) injections administered to rat or mouse dams resulted in sensorimotor gating dysfunction, increased anxiety, impairment of social interactions, and other abnormal behaviors in newborns, showing that an inflammatory response is sufficient to cause behavioral abnormalities associated with brain dysfunction [135-137]. IFN- $\gamma$  is one of the cytokines that is known to be released in the fetal brain during infection [109, 111, 138]. As *Neurog2* is known to generate glutamatergic neurons while

suppressing the generation of GABAergic neurons, the downregulation of *Neurog2* expression due to IFN- $\gamma$  may lead to an abnormal ratio of glutamatergic to GABAergic neurons in the mature brain. An imbalance of glutamate and GABA has been reported to be a major cause of various neurological diseases including autism, Rett syndrome, schizophrenia, and mood disorders [139-141]. In this regard, it is interesting to note that the glutamate to GABA ratio was reduced whereas the level of IFN- $\gamma$  was increased in the plasma of autistic patients compared with healthy subjects, suggesting a link between IFN- $\gamma$  and brain abnormalities [139]. Although it is not yet clear what the consequences of the IFN- $\gamma$ -mediated dysregulated neuronal differentiation during embryonic development are, the findings from this chapter may provide an interesting starting point for understanding the role(s) of IFN- $\gamma$  during brain development, and ultimately, the neurodevelopmental disorders caused by congenital infections.



## **CHAPTER VI**

# **Search for Cis-acting Sequence Involved in the IFN- $\gamma$ -mediated Inhibition of *Neurog2* Expression**

# 1. Background

*Neurog2*, a bHLH transcription factor, is an atonal proneural gene, which is expressed in the brain and spinal cord throughout development. Its expression is restricted to the dorsal telencephalon in the brain and it is known to play a role in the differentiation of early-born neurons in the cerebral cortex [128, 129, 142, 143]. It is an essential factor for initiating the neuronal differentiation of NPCs, and it is sufficient to generate neurons from mouse embryonic stem cells [130-132]. Although *Neurog2* has been known as a key factor in the determination of neural cell types, and its expression pattern is tightly regulated, only a few factors are known to regulate its expression.

The regulation of proneural genes occurs mostly at the transcriptional level. The transcription factors that are known to regulate *Neurog2* expression include the paired-box transcriptional activators, Pax3 and Pax6, and the transcriptional repressor, Hes1. Pax3 has been studied widely in the context of cell migration, neurogenesis, cardiogenesis, and melanocyte stem cell differentiation. Pax3 mutant mice exhibit premature neurogenesis with abnormal expression of *Hes1* and *Neurog2* [133, 144]. By binding to the promoters of *Hes1* and *Neurog2*, Pax3 activates the expression of *Hes1* during the early stages of neurogenesis to promote the proliferation of NPCs, and then activates *Neurog2* at a later stage to initiate neuronal differentiation. Another homeodomain protein, Pax6, plays a key role in patterning the neural tube in response to Shh, and it is expressed in the spinal cord and lateral cortex. Pax6 activates *Neurog2* expression by binding directly to the E1 enhancer element [145]. *Hes1*, a Notch target gene, is expressed in NPCs in an oscillating pattern with *Neurog2* in the developing telencephalon [146, 147]. The expression of *Neurog2* leads to Notch ligand expression, which activates the Notch signaling pathway in neighboring cells, producing Hes1. Hes1 then inactivates *Neurog2* via direct promoter binding, blocking its transcription. FGF-2 has also been reported to negatively regulate *Neurog2* expression by activating the Notch pathway in the dorsal root ganglia [148].

Recently, a transcription factor, RP58 (ZNF238), has also been implicated in the activation of *Neurog2*. RP58 is highly conserved (>95%) in humans and mice, and it is expressed in NPCs and

neurons. The neural-specific RP58 KO mice show defects in brain development with decreased neurogenesis, suggesting a role for RP58 in early corticogenesis. RP58 binds directly to the promoter region of *Neurog2* and represses its expression in differentiating neurons. It also represses the expression of *NeuroD1* in INPs, controlling the timing and the extent of neuron generation [149]. The key player in the Wnt pathway,  $\beta$ -catenin, has also been found to increase the *Neurog2* promoter activity in 293 cells, although the direct binding site has not yet been experimentally determined [150].

The expression of *Neurog2* is known to be regulated primarily at its transcription level. However, no definite link has been suggested between the inflammatory signaling pathway and the regulation of *Neurog2*. In this chapter, the mechanism by which IFN- $\gamma$  inhibits *Neurog2* transcription was investigated using a reporter plasmid under the control of the *Neurog2* promoter. The promoter activity was tested in various experimental settings using recombinant IFN- $\gamma$  and a cell line overexpressing STAT1 by employing a transient or stable transfection method. In every case, the activation of STAT1 led to the reduction in the *Neurog2* promoter activity. The data from the experiments involving a translational inhibitor and different forms of the *Neurog2* promoter suggested that the IFN- $\gamma$ -mediated negative regulation of *Neurog2* might be achieved by complex mechanisms, which may require *de novo* protein synthesis, rather than by simple promoter binding.

## 2. Results

### 2.1 Sequence Analysis of the *Neurog2* Promoter

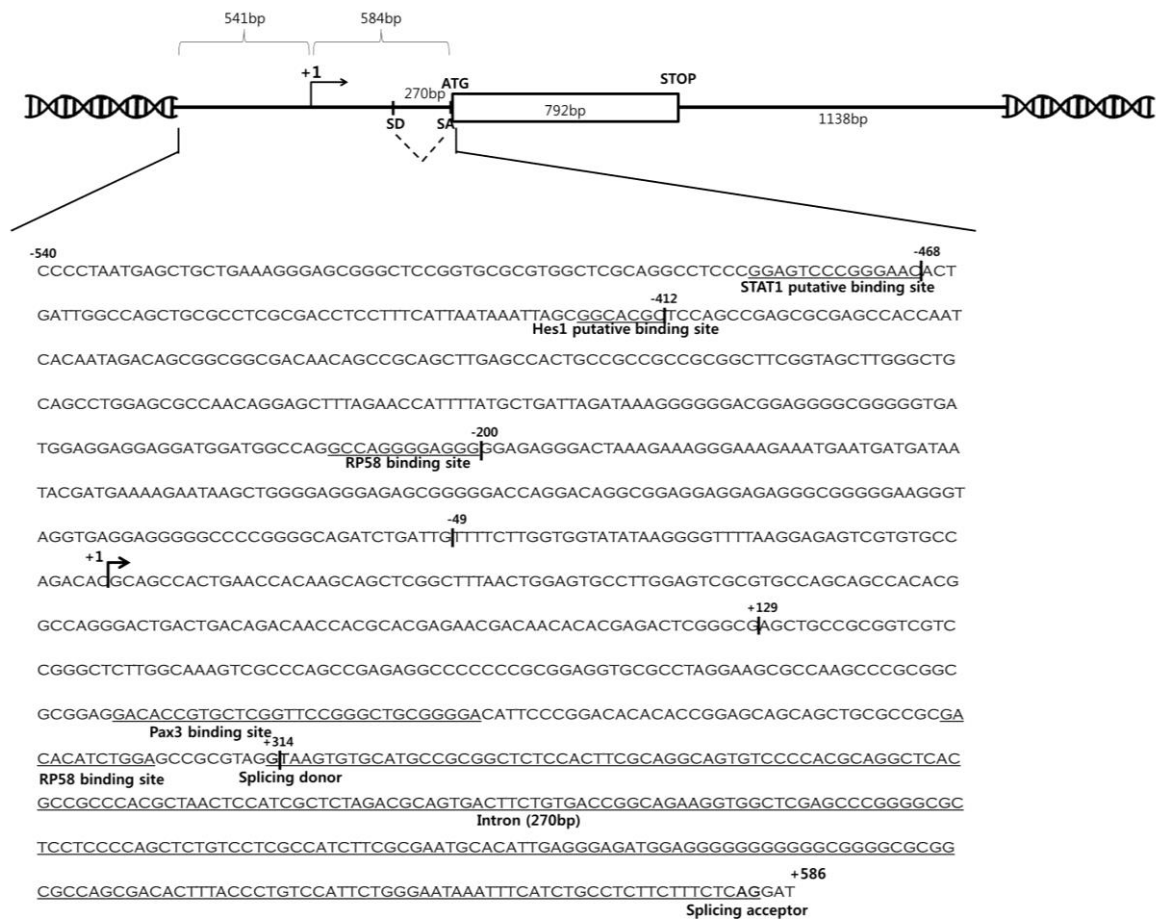
The *Neurog2* genome comprises a 5' untranslated region (UTR) including an intron, *Neurog2* cDNA, and a 3' UTR. *Neurog2* mRNA is spliced at splicing donor and splicing acceptor sites, which are located at +314 and +581, respectively. The STAT1 transcription factor can bind directly to a specific DNA sequence known as the IFN- $\gamma$  activating sequence (GAS); therefore, the *Neurog2* promoter was analyzed using the TRANSFAC database to determine whether it contains a functional GAS. One putative STAT1 binding site (5'-ggagtcccgggaa-3') was found 468 bp upstream from the

transcription start site. To investigate whether the IFN- $\gamma$ -mediated negative regulation of *Neurog2* expression occurs at the level of transcriptional initiation, the minimal essential *Neurog2* promoter containing the 5' upstream region of the *Neurog2* gene from -540 to +586 bp was cloned (Fig. VI-1) [133].

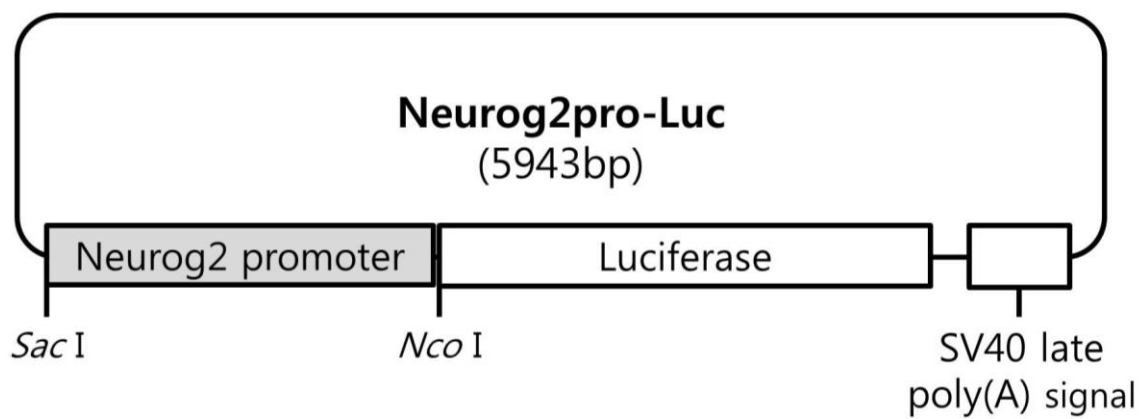
## **2.2 Effects of IFN- $\gamma$ on the activity of the transiently transfected *Neurog2* promoter**

To perform a promoter reporter assay, a luciferase expression plasmid under the control of the *Neurog2* promoter (Neurog2pro-Luc) was constructed (Fig. VI-2). First, primary NPCs were electroporated with the Neurog2pro-Luc and after 2 days, they were differentiated with 50 ng/mL IFN- $\gamma$  for 12 hr. The level of luciferase activity increased almost two-fold upon differentiation and this upregulation was completely inhibited when IFN- $\gamma$  was added (Fig. VI-3A). When NPCs were treated with 10, 50, or 100 ng/mL IFN- $\gamma$ , the *Neurog2* promoter activity was decreased by 13.5%, 47.7%, and 59%, respectively, demonstrating a dose-dependent response (Fig. VI-3B). These results indicate that the *Neurog2* promoter activity is increased upon differentiation, while IFN- $\gamma$  inhibits this up-regulation at the promoter level.

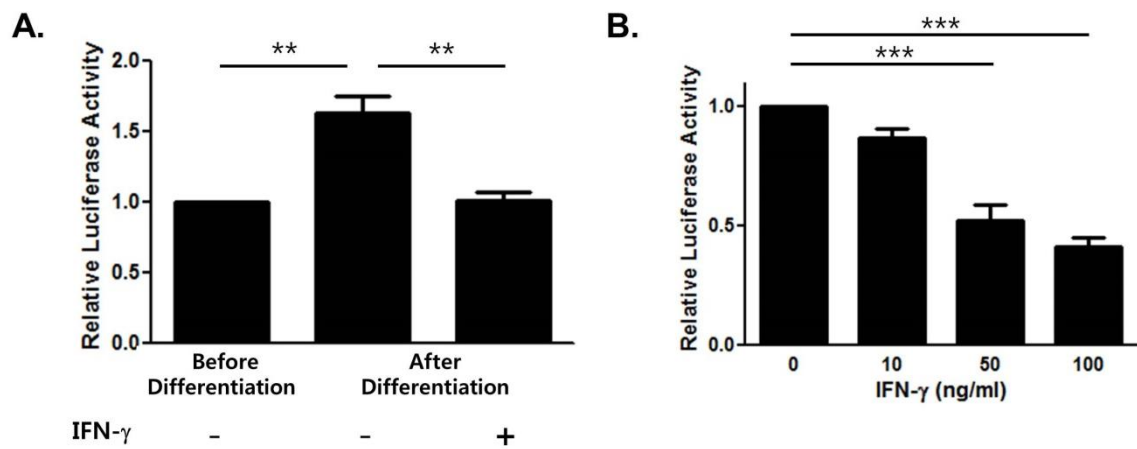
The effects of IFN- $\gamma$  treatment on the *Neurog2* promoter activity were also tested in NIH3T3 cells, which are commonly used in luciferase assays and known to be responsive to murine IFN- $\gamma$ . NIH3T3 cells were treated with IFN- $\gamma$  for 1 day, and the level of *Neurog2* mRNA was measured (Fig. VI-4A). Similar to the results obtained from primary NPCs, IFN- $\gamma$  treatment reduced *Neurog2* mRNA expression by approximately 60%. When NIH3T3 cells were transfected with Neurog2pro-Luc and treated with 10, 50, or 100 ng/mL IFN- $\gamma$  for 1 day, there was 25.1%, 35.3%, and 37% reduction in the promoter activity, respectively (Fig. VI-4B). This data show that the downregulation of *Neurog2* expression and the promoter activity upon IFN- $\gamma$  treatment in NIH3T3 cells were similar to the data observed in primary NPCs, validating the use of NIH3T3 cells in further experiments.



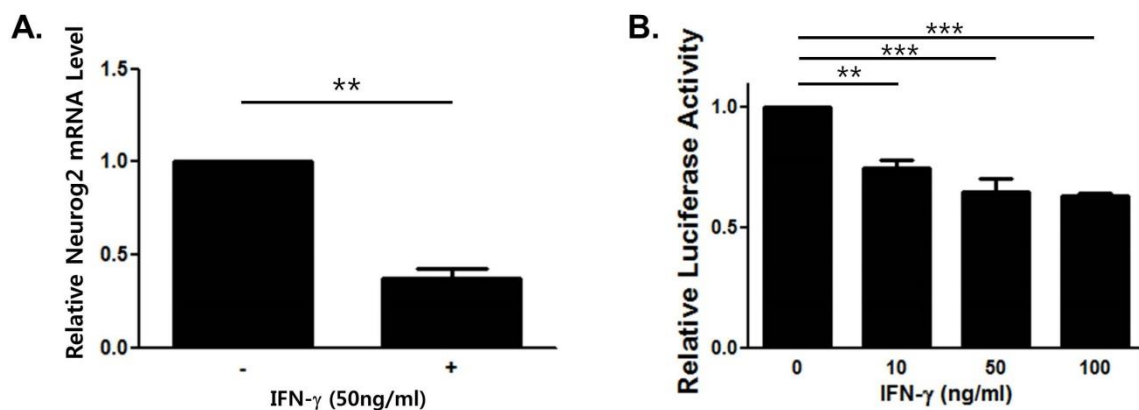
**Figure VI-1. *Neurog2* promoter sequence.** The *Neurog2* genome comprises of the promoter region (1125bp) including a 5' UTR with an intron, *Neurog2* cDNA (792 bp), and a 3' UTR (1138 bp). *Neurog2* mRNA is spliced at splicing donor site located at +314 and splicing acceptor site at +581. The region between 540 bp upstream and 586 bp downstream from the transcription start site (+1), which includes the 270bp intron is known as the minimal *Neurog2* promoter. Experimentally proven or putative binding sequences of different regulators in the *Neurog2* promoter are indicated. SD, splicing donor; SA, splicing acceptor.



**Figure VI-2. Schematic diagram of the Neurog2pro-Luc.** A *SacI*/*NcoI* fragment that lies between 540bp upstream and 586bp downstream from the *Neurog2* transcription start site was cloned into the pGL3-Basic plasmid (promega) using PCR. The diagram is not to scale.



**Figure VI-3. IFN- $\gamma$  downregulates the activity of the *Neurog2* promoter in primary NPCs. (A, B)** Primary NPCs were isolated from E14.5 embryonic brains and electroporated with pNeurog2-Luc. After 1 day, cells were differentiated with or without IFN- $\gamma$  for 12 hr and subjected to a luciferase reporter assay. The relative luciferase activity is shown. \*\*P < 0.01; \*\*\*P < 0.001.



**Figure VI-4. IFN- $\gamma$  downregulates the expression of *Neurog2* mRNA and the activity of the *Neurog2* promoter in NIH3T3 cells.** NIH3T3 cells were transfected with the pNeurog2-Luc. After 3 hr, IFN- $\gamma$  was added with fresh medium for 1 day. **(A)** Total RNA was isolated, and the level of *Neurog2* mRNA was measured by qRT-PCR. **(B)** Cell lysates were prepared and subjected to a luciferase reporter assay. The relative luciferase activity is shown. \*\*P < 0.01; \*\*\*P < 0.001.

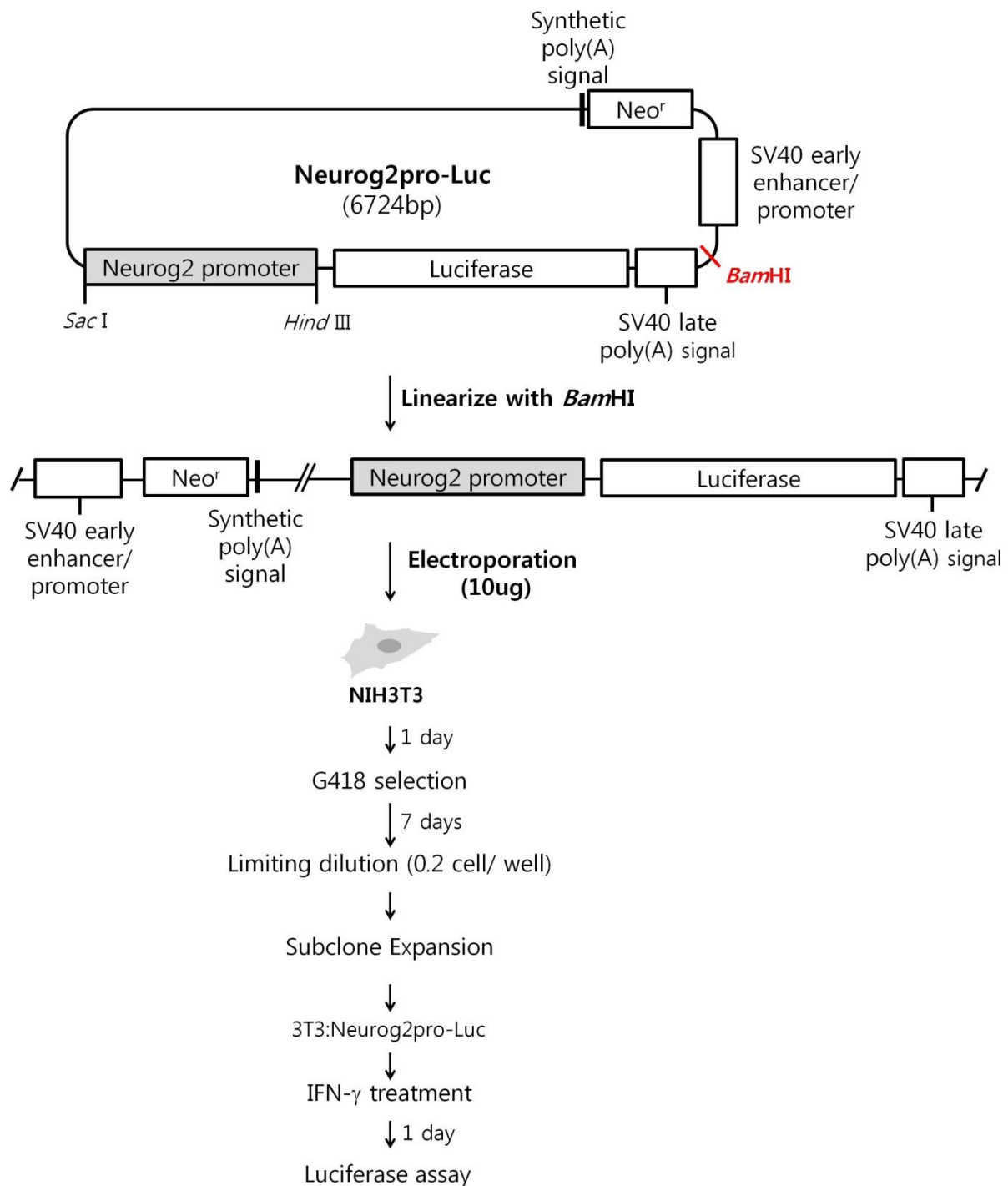


### **2.3 Effects of IFN- $\gamma$ on the activity of the stably transfected *Neurog2* promoter**

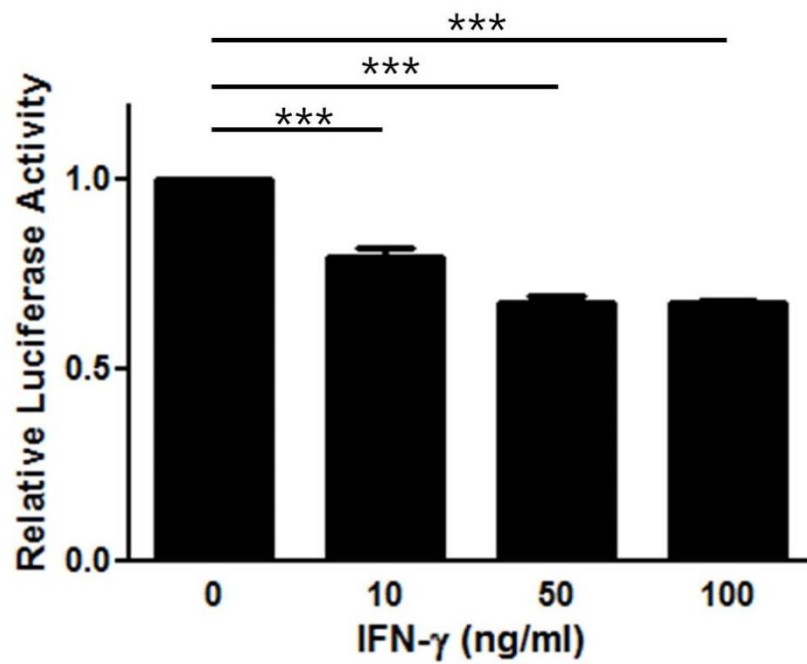
The results obtained using transiently transfected reporter plasmids could be fundamentally different from those observed in nature, because the gene is normally present in the chromosome as a single copy or very few copies, whereas multiple extrachromosomal copies of the promoter are present in the case of transient transfection. Therefore, the effects of IFN- $\gamma$  on a stably integrated *Neurog2* promoter were investigated. The Neurog2pro-Luc-Neo<sup>R</sup> plasmid that contains a neomycin-resistance gene was constructed and linearized at the *Bam*HI restriction site to facilitate the efficient integration of the plasmid DNA into the cellular chromosome (Fig. VI-5). The linearized Neurog2pro-Luc-Neo<sup>R</sup> plasmid was electroporated into NIH3T3 cells and subjected to G418 selection for 1 week. Using the serial dilution method, the cells were plated onto 96-well plates, and eight drug-resistant cell clones were obtained. The clone with the highest basal luciferase activity was selected, expanded, and designated as 3t3: Neurog2pro-Luc. The 3t3: Neurog2pro-Luc cells were treated with IFN- $\gamma$  for 1 day and subjected to a luciferase assay. IFN- $\gamma$  treatment reduced the *Neurog2* promoter activity by approximately 40% (Fig. VI-6). This result is in a good agreement with the data obtained from the transient transfection assay, suggesting that the transient transfection method is suitable for further experiments.

### **2.4 Effects of STAT1 overexpression on the activity of the *Neurog2* promoter**

The IFN- $\gamma$ -mediated downregulation of *Neurog2* expression is dependent on the JAK/STAT1 pathway; therefore, the effects of STAT1 expression on the *Neurog2* promoter activity were investigated. A cell line (3T3-STAT1ca) that overexpresses a constitutively active form of STAT1 (STAT1ca) was generated using a retroviral vector. STAT1ca contains two cysteine residues in the place of Ala-656 and Asn-658, which allows the formation of a dimer via a disulfide bond [18] (Fig.



**Figure VI-5. Overall procedure used for the stable transfection of the pNeurog2-Luc plasmid.** A *Sac*I/*Hind*III fragment that lies between 540 bp upstream and 586 bp downstream from the transcription start site was cloned into pGL4.17 plasmid using PCR and designated as Neurog2pro-Luc-Neo<sup>R</sup>. The plasmid was linearized with *Bam*HI. 10  $\mu$ g of the linearized plasmid was electroporated into NIH3T3 cells followed by G418 selection. The G418-resistant cell clone with the highest basal luciferase activity was selected, expanded, and designated as 3T3:Neurog2pro-Luc. The diagram is not to scale.



**Figure VI-6. Effects of IFN-γ on the expression of the integrated luciferase gene.** The selected NIH3T3 cells stably expressing luciferase gene under the control of the *Neurog2* promoter (3T3:Neurog2pro-Luc) were treated with different concentrations of IFN-γ for 1 day and subjected to a luciferase assay. The relative luciferase activity is shown. \*\*\*P < 0.001.

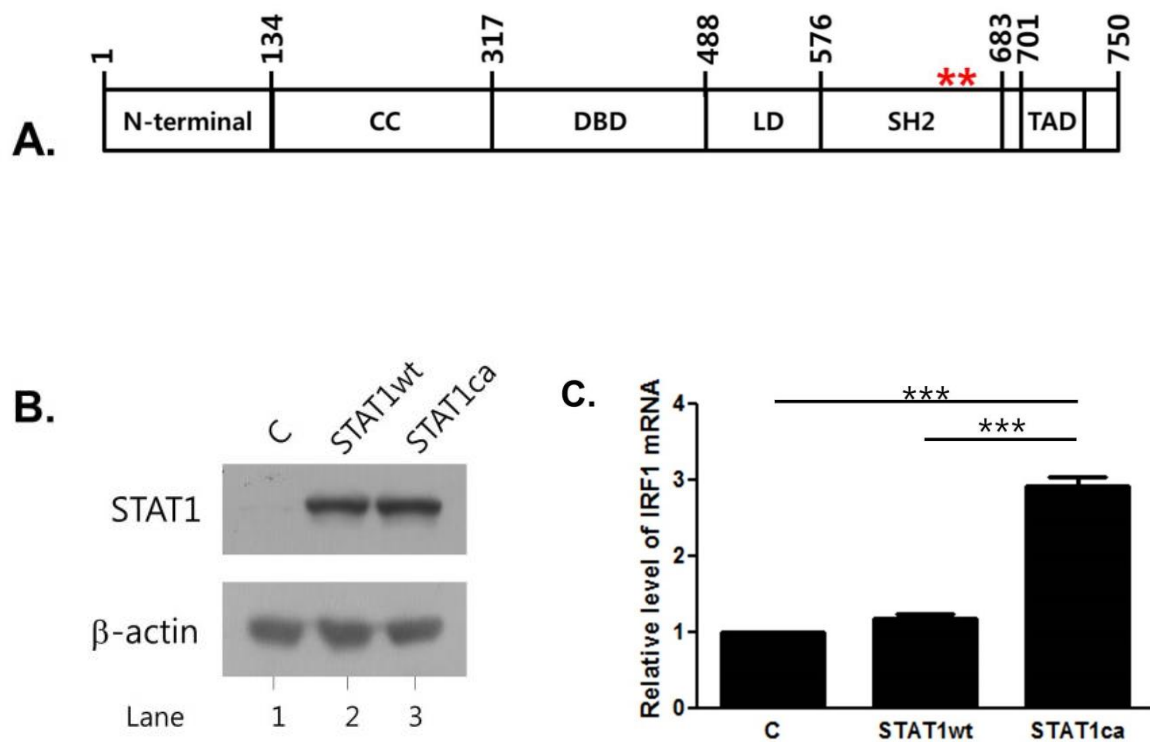
VI-7A). The expression and functionality of STAT1ca were confirmed by western blotting, and qRT-PCR that measured the mRNA levels of one of the STAT1 target genes, *Interferon regulatory factor 1* (*IRF1*) (Fig. VI-7B, C). STAT1 was not expressed in the control cells, whereas it was detected in the wild-type STAT1-expressing cells (3T3-STAT1wt) and 3T3-STAT1ca. Compared with the control and 3T3-STAT1wt, the level of *IRF1* mRNA in the 3T3-STAT1ca was approximately three times higher, indicating that STAT1ca is indeed constitutively active and able to activate downstream target genes. The *Neurog2*pro-Luc plasmid was transfected into the 3T3-control or -STAT1ca cells for 1 day, and the total proteins were extracted and subjected to a luciferase assay (Fig. VI-8A). The expression of STAT1ca did not affect the level of luciferase activity in promoter-less (mock) vector-transfected cells. Similar to the experiment performed with IFN- $\gamma$ , the luciferase activity level in the 3T3-STAT1ca was approximately half of that in the control cells, suggesting that STAT1 may play a key role in the IFN- $\gamma$ -mediated downregulation of *Neurog2* promoter activity. An IFN- $\gamma$  overexpressing cell line was also tested, however the stable expression of IFN- $\gamma$  affected the level of luciferase activity in mock vector-transfected cells, thus excluded for the further experiments (Fig. VI-8B).

## 2.5 Search for the IFN- $\gamma$ -responsive regulatory element in the *Neurog2*

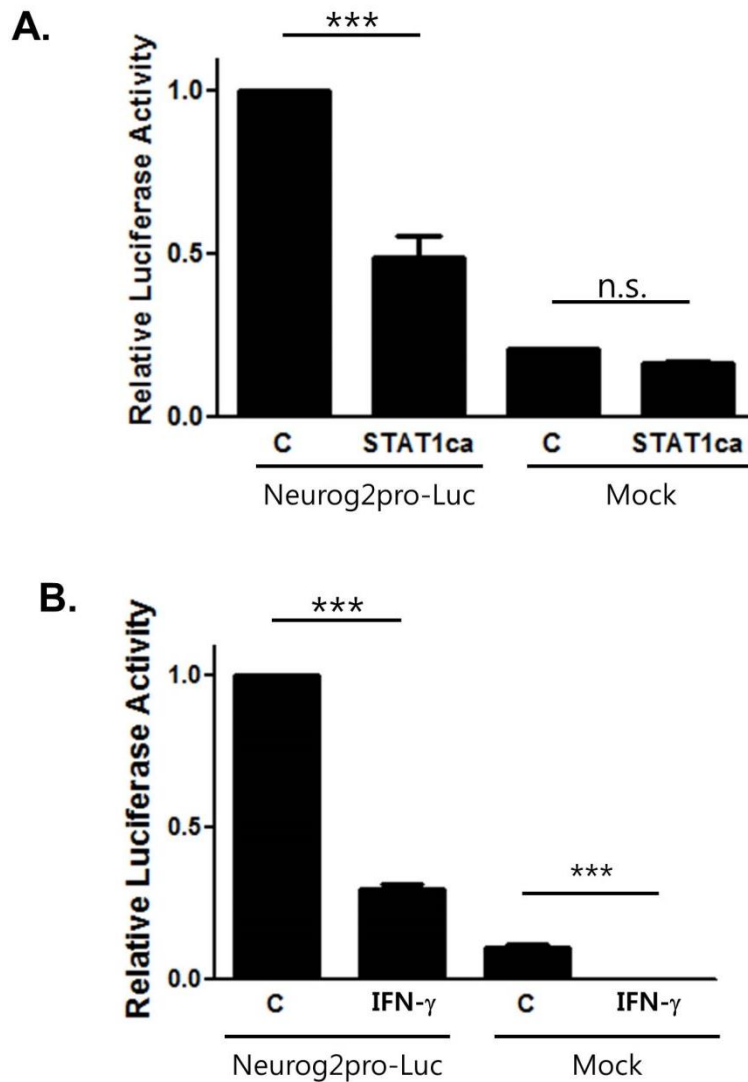
### Promoter

Eight different promoters with deletion or mutation were constructed to identify the IFN- $\gamma$ -responsive regulatory element in the *Neurog2* promoter (Fig. VI-9). Along with one promoter that lacks the putative STAT1 binding site (Fig. VI-9 #2), six other short forms of the *Neurog2* promoter were constructed as STAT1 is known to be permissive for mismatches and can bind to unique DNA sequences by interacting with other proteins [151]. A mutant form containing an inactive splicing site (Fig. VI-9 #8) was also constructed to determine whether the splicing event is crucial for *Neurog2* expression.

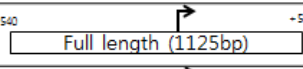
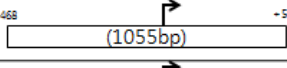
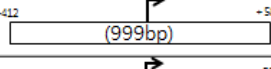
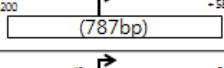
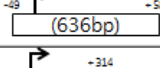
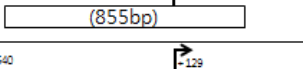
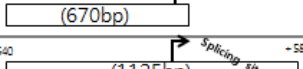
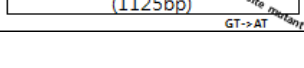
The 3T3-control or 3T3-STAT1ca cells were transfected with the appropriate luciferase



**Figure VI-7. Construction of a constitutively active form of STAT1.** (A) The domain structure of STAT1 protein is shown. The residues affected by mutation are shown in red. The tyrosine phosphorylation site is present at amino acid 701. For the constitutively active form of STAT1, cysteine residues were introduced into Ala-656 and Asn-658. CC, coiled coil domain; DBD, DNA binding domain; LD, linker domain; SH2, Src homology 2 domain; TAD, transcriptional activation domain. (B) Total cellular proteins were prepared from the control, STAT1wt-, and STAT1ca-overexpressing NIH3T3 cells, and then subjected to western blotting using antibodies to total STAT1 and  $\beta$ -actin. (C) Total RNA from the control, STAT1wt-, and STAT1ca-overexpressing NIH3T3 cells were isolated, and *IRF1* mRNA levels were measured by qRT-PCR.



**Figure VI-8. Effects of STAT1ca expression on the activity of the *Neurog2* promoter.** (A) The Neurog2pro-Luc or the promoter-less (Mock) plasmid was transfected into the control or STAT1ca-overexpressing NIH3T3 cells. After 1 day, the cell lysates were subjected to a luciferase reporter assay. The relative luciferase activity is shown. (B) The Neurog2pro-Luc or Mock plasmid was transfected into the control or IFN- $\gamma$ -overexpressing NIH3T3 cells. After 1 day, the cell lysates were subjected to a luciferase reporter assay. The relative luciferase activity is shown. \*\*\* $P < 0.001$ ; n.s., not significant.

				Relative Luciferase Activity	Fold change	Statistical significance
[#1]	-540/+586	 Full length (1125bp)	C	1	-1.9	***
			STAT1ca	0.52±0.09		
[#2]	-468/+586	 (1055bp)	C	0.94±0.15	-2.4	***
			STAT1ca	0.40±0.08		
[#3]	-412/+586	 (999bp)	C	0.84±0.11	-2.2	***
			STAT1ca	0.38±0.03		
[#4]	-200/+586	 (787bp)	C	0.95±0.16	-2.4	***
			STAT1ca	0.40±0.08		
[#5]	-49/+586	 (636bp)	C	0.98±0.17	-2.6	***
			STAT1ca	0.37±0.05		
[#6]	-540/+314	 (855bp)	C	1.17±0.16	-2.2	***
			STAT1ca	0.53±0.08		
[#7]	-540/+129	 (670bp)	C	1.24±0.17	-2.3	***
			STAT1ca	0.54±0.11		
[#8]	Splicing site mutant	 (1125bp)	C	0.81±0.15	-2.1	***
			STAT1ca	0.38±0.10		

**Figure VI-9. Effects of STAT1 overexpression on different forms of the *Neurog2* promoters.**

Schematic diagrams are shown for eight different forms of the *Neurog2* promoter. The arrow indicates the transcription start site. The control or STAT1ca-overexpressing NIH3T3 cells were transfected with the respective reporter plasmids. After 1 day, the cell lysates were subjected to a luciferase reporter assay. The relative luciferase activity, fold change, and statistical significance are shown.

\*\*\*P<0.001.

plasmid and a luciferase assay was performed after 1 day. There were no statistically significant differences in the basal activities of all the promoters tested (Fig. VI-9). Interestingly, the inhibitory effect of STAT1ca on the *Neurog2* promoter activity was observed in all eight forms of the promoter, with a 48–62% reduction. These results suggest that there might not be a cis-acting sequence interacting with STAT1 within the -540 to +586 bp region of the *Neurog2* genome, and that the reduction might be achieved via complex mechanisms, and not by simple promoter binding.

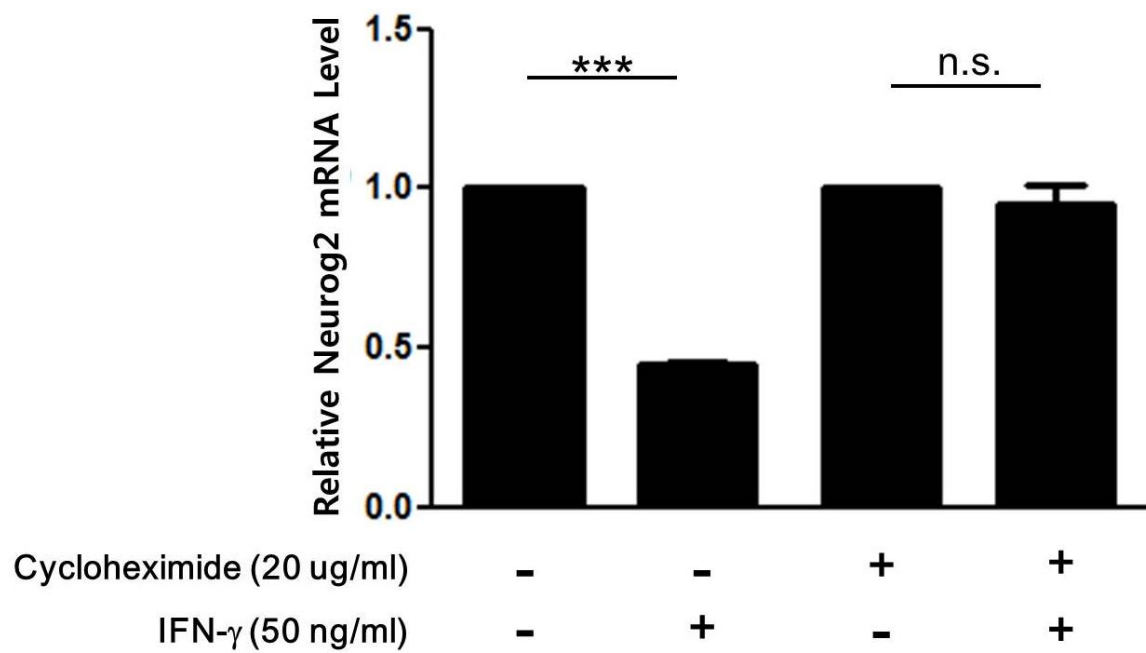
## **2.6 IFN- $\gamma$ -mediated inhibition of *Neurog2* expression requires *de novo* protein synthesis**

Many proteins are expressed upon STAT1 activation, and therefore, it is possible that STAT1 induces the expression of other transcription factors, which might in turn regulate the expression of *Neurog2*. To test whether the synthesis of other proteins is required for the regulation of *Neurog2* expression, primary NPCs were treated with a translational inhibitor, cycloheximide, and differentiated with or without IFN- $\gamma$ . IFN- $\gamma$  treatment resulted in the reduction of *Neurog2* expression by 55%, while this inhibitory effect was completely reversed upon cycloheximide treatment (Fig. VI-10). This data indicates that the IFN- $\gamma$ -mediated suppression of *Neurog2* expression may require *de novo* protein synthesis.

## **3. Discussion**

As described in the previous chapter, IFN- $\gamma$  treatment downregulates the expression of *Neurog2* that plays a crucial role in neuronal differentiation. To understand the precise relationship between IFN- $\gamma$  and neurogenesis, the molecular mechanisms underlying the IFN- $\gamma$ -mediated downregulation of *Neurog2* expression were investigated. The *Neurog2* promoter was cloned and its





**Figure VI-10. *De novo* protein synthesis is required for the IFN- $\gamma$ -mediated downregulation of *Neurog2*.** NPCs were differentiated in the presence of 20  $\mu$ g/mL cycloheximide and 50 ng/mL IFN- $\gamma$  for 12 hr. Total RNA was isolated and the *Neurog2* mRNA level was measured using qRT-PCR. \*\*P < 0.01; \*\*\*P < 0.001; n.s., not significant.

activity was measured in various experimental conditions by activating the STAT1 signaling pathway using different methods. In both the IFN- $\gamma$  treated and STAT1ca-expressing cells, the activity of the *Neurog2* promoter was nearly half of that in the control cells. To discern any possible artifacts that might result from the transient transfection assay where the plasmid is present in its extrachromosomal form, the reporter plasmid was integrated into the cellular chromosome. The activity of the integrated promoter was reduced to a similar level upon IFN- $\gamma$  treatment, indicating that there is approximately two-fold contribution of IFN- $\gamma$  to the *Neurog2* promoter activity.

To identify the STAT1 regulatory element in the *Neurog2* promoter, deletion mutations were introduced into the *Neurog2* promoter, and the effects of IFN- $\gamma$  on their activity were tested. Interestingly, the IFN- $\gamma$ -mediated inhibition of the *Neurog2* promoter activity was not rescued in any of the deletion forms. Moreover, there was an approximately five-fold difference in the magnitude of reduction in the level of the *Neurog2* mRNA expression and the promoter activity. Several other studies have also presented the similar case that showed up to ten-fold magnitude differences between the level of mRNA expression and the promoter activity [152-154]. These results could be due to differences between the experimental system and the natural condition as the transfection of a reporter plasmid might not ensure that the promoter is regulated in a natural way.

It is also highly likely that regions outside the *Neurog2* promoter are affected by IFN- $\gamma$ . In the spinal cord, it has been reported that the regulation of *Neurog2* can be achieved via cis-regulatory regions located thousands of base pairs upstream or downstream from the transcription start site [155]. These enhancers are highly conserved in different species, suggesting its role in gene regulation. The upstream regulator of *Neurog2*, Pax6, was found to interact with one of the enhancers, E1, which is located more than 7 kbp upstream from the transcription start site [145]. Retinoic acid response elements and a Gli binding site were also found in the E3 enhancer element, which is located more than 3 kbp downstream from the transcription start site [155, 156]. Another possible mechanism may be via epigenetic control. There have been yet no studies of the effects of IFN- $\gamma$  on this type of regulation, but IFN- $\gamma$  may contribute to changes in methylation or acetylation patterns of *Neurog2* as

the gene expression pattern in progenitor cells is often regulated by epigenetic factors [16, 17]. Changes in mRNA stability could also have led to the reduction in *Neurog2* expression, although there have been yet no studies reported. In addition, several other factors might play a role in the downregulation of *Neurog2* expression upon IFN- $\gamma$  treatment according to the data from cycloheximide experiment. As IFN- $\gamma$  treatment did not change the expression pattern of the factors those were reported to regulate *Neurog2* expression such as Pax3, Pax6, Hes1,  $\beta$ -catenin, and RP58 in primary NPCs (data not shown), other novel factors are believed to play a role. Overall, the data from this chapter suggested that the IFN- $\gamma$ -mediated reduction of *Neurog2* expression might involve complex mechanisms rather than an interaction between the promoter and one particular DNA-binding protein. Further investigations are required to determine the exact mechanism by which IFN- $\gamma$  abrogate the expression of *Neurog2* in primary NPCs.

## **CHAPTER VII**

### **Conclusion**

The primary aim of this thesis research was to determine the roles of stress-related factors in the initiation of neurogenesis. As the first step, I screened five genes, whose expression levels were previously known to be regulated under stressed condition. Their effects on the neuronal differentiation of NPCs were investigated using primary NPCs. The two factors with strong negative effects on neurogenesis, GSK3 $\beta$  and IFN- $\gamma$ , were selected and further characterized *in vitro* as well as *in vivo* using the UIGD technique.

GSK3, which has been reported to be upregulated in the case of prenatal alcohol exposure, is considered to be the master regulator of neurogenesis. However, the exact roles of the two isoforms of GSK3 have not been made clear. In this study, the functions of both isoforms were studied in primary NPCs and in the embryonic brain using various techniques such as chemical inhibitor, overexpression, and shRNAs. Through these experiments, it was revealed that only GSK3 $\beta$ , but not GSK3 $\alpha$ , inhibits embryonic neurogenesis. Furthermore, by treating rapamycin to GSK3 $\alpha$ - or GSK3 $\beta$ -knock-down cells, it was suggested that mTORC1 might differentially regulate the activity of GSK3 $\alpha$  and GSK3 $\beta$ . This is the first study to clarify the different roles of GSK3 $\alpha$  and GSK3 $\beta$  in the context of embryonic neurogenesis, as well as the relationship between mTORC1 and GSK3 in primary NPCs. These findings are believed to be useful for understanding the molecular function of GSK3 in general, as well as facilitating the development of new and effective therapeutic targets for the many diseases in which GSK3 is known to play a role.

Another detailed biological investigation was carried out on IFN- $\gamma$  which has been reported as the major cytokine released in the embryonic brain upon prenatal infection. Prior to this study, the role of IFN- $\gamma$  in embryonic neurogenesis and its downstream mechanism have been elusive. In this research, the function of IFN- $\gamma$  in the embryonic brain was tested for the first time, and the obtained results demonstrated that IFN- $\gamma$  has a negative role in neuronal differentiation *in vivo*. Moreover, it was found that IFN- $\gamma$  inhibits the neuronal differentiation of primary NPCs by reducing the NPC-neuronal transition via the JAK/STAT1 pathway, and repressing one of the proneural genes, *Neurog2*. To the best of my knowledge, this is the first study to suggest a role of IFN- $\gamma$  in the regulation of

proneural gene expression in differentiating NPCs. These findings may provide an interesting starting point for understanding the role(s) of IFN- $\gamma$  during brain development, and ultimately, in the neurodevelopmental disorders caused by congenital infections.

Many case reports have suggested that stress during pregnancy can significantly affect brain development in children. Gestational exposure to alcohol has been reported to affect multiple stages of brain development including neuronal differentiation, proliferation, migration and gliogenesis, leading to many neurological defects in newborn children, such as microcephaly, mental retardation, lack of focus and delayed development, which are collectively known as fetal alcohol spectrum disorders (FASDs) [157]. In the case of neurogenesis, alcohol has been known to inhibit it, reducing the number of cortical neurons; however, clear studies of the mechanism are lacking [158-162]. When a pregnant dam is exposed to alcohol, the alcohol crosses the placenta and directly affects gene expression in the fetus. Interestingly, several studies have reported that the PI3K/AKT/mTOR pathway is inhibited upon exposure to ethanol in mouse myocytes, rat hearts and human neuronal cells [163-165], while the activity of GSK3 has been reported to be increased in ethanol-treated rat cortical neuroblasts, and the hippocampus of adolescent (postnatal day 23) mice prenatally exposed to alcohol [166, 167]. According to these studies and the results from my thesis, one of the possible pathogenesises of FASDs would be due to the inhibition of the mTORC1 signaling pathway upon ethanol exposure, which would in turn activate GSK3 $\beta$ , leading to strong inhibition of embryonic neurogenesis. Although further *in vivo* studies are required, the findings from this thesis may provide a stepping stone to future mechanistic studies on FASDs.

Congenital infection is another prevalent cause that is thought to be responsible for brain developmental defects. Lymphocytic choriomeningitis virus, influenza virus, rubella virus, human cytomegalo virus, *Campylobacter rectus* and *Toxoplasma gondii* are some examples of the pathogens that are reported to cause severe neurological defects in infants who have survived congenital infection [57-62]. However, the exact mechanism of pathogenesis due to congenital infection has not yet been clearly defined. Several studies have suggested that the inflammatory response is the cause of neurological disorders [69], and IFN- $\gamma$  has been known to be increased upon infection in the fetal

brain [109, 111, 138]. Moreover, the elevated concentrations of IFN- $\gamma$  during the second trimester of pregnancy are significantly associated with an increased risk of a child being diagnosed with autism [168]. Interestingly, the level of plasma IFN- $\gamma$  has been found to be induced in autistic patients, suggesting a relationship between IFN- $\gamma$  and neurological disorders [169]. According to the results obtained in the present study, the abnormal expression of IFN- $\gamma$  in embryonic NPCs inhibits the expression of a major proneural gene, *Neurog2*. The downregulation of *Neurog2* not only inhibits the neuronal differentiation of NPCs, but may also lead to weak neuronal circuits as well as imbalances in the glutamatergic neurons and GABAergic neurons, which are the two main causes of neurodevelopmental diseases [139-141]. The results reported in this thesis may provide insights into the pathogenesis of fetal brain dysfunction after infection.

In this thesis, I investigated the roles of genes that are known to be upregulated during stressed conditions in embryonic neurogenesis, which occurs at the earliest stage of brain development. The results of this study clearly suggest GSK3 $\beta$  and IFN- $\gamma$  as strong negative controllers of embryonic neurogenesis. As the extent of neurogenesis during the early period of development is critical for shaping brain functions, the findings from this study may contribute to the understanding of basic brain development as well as the pathogenesis of many congenital neurological disorders. Although the experimental design employed in this research was efficient and yielded insightful data, further experiments are required to provide more relevant answers in the context of congenital brain defects. It would be valuable to determine whether the key behavioral defects those seen in FASDs, schizophrenia, or autism models (such as cognitive dysfunctions, increased anxiety level, or deficits in social interaction) appear when the activity of GSK3 $\beta$  or IFN- $\gamma$  is induced in the fetal brain, and whether these abnormal behaviors are rescued when the activity of GSK3 $\beta$  or IFN- $\gamma$  is blocked. Such results would provide powerful clues that can be applied in the development of a preventative drug or a treatment for brain disorders due to developmental defects.

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## 국문초록

태아가 스트레스에 노출될 경우 태어난 후에 심각한 신경 질환으로 이어질 수 있는 것이 밝혀져 있다. 많은 인자가 스트레스 상황과 관련이 있는 것으로 알려졌지만, 뇌 발달에서 그 역할이 명확하게 조사된 바는 없다. 이 연구는 실생활에서 흔히 일어나는 태아기 스트레스 중, 알코올 섭취와 바이러스 감염에 의해 태아 뇌에서 활성화된다고 알려진 다섯 개 유전자 (Nrf2, GSK3 $\alpha$ , GSK3 $\beta$ , IFN- $\gamma$ 와 SC4MOL)를 선정하고, primary embryonic day (E) 14.5 생쥐 신경 전구세포 (neural progenitor cells: 이하 NPCs)에서 그 영향을 조사하는 것으로 시작되었다. 레트로바이러스를 사용하여 각각의 유전자를 NPCs에서 과발현시킨 후, TuJ1 염색을 통해 분화된 신경세포의 비율을 분석하였다. GSK3 $\beta$ 와 IFN- $\gamma$ 는 과발현되었을 경우 TuJ1-양성 (TuJ1<sup>+</sup>) 세포가 크게 감소하는 것을 관찰하였다. 이는 이 두 유전자가 신경세포의 분화를 강하게 억제한다는 것을 의미하는 것으로서, 이후 다양한 기법들을 이용하여 신경세포 분화에서 이 두 인자의 역할과 기전을 조사하였다.

GSK3는 NPCs의 증식 및 분화를 조절하는 중요한 역할을 하는 것으로 잘 알려져 왔으나 두 개 동형단백질(isoform)인 GSK3 $\alpha$ 와 GSK3 $\beta$  각각의 역할에 대해서는 논란이 있었다. 따라서 이 연구에서는 신경 분화의 과정에서 GSK3 $\alpha$  및 GSK3 $\beta$ 의 기능에 차이가 있는지를 조사하는 데 초점을 두었다. GSK3 억제제 (SB216763) 처리 시, NPCs의 TuJ1<sup>+</sup> 신경세포의 증가가 관찰되었는데 이는 GSK3가 신경세포 분화를 억제한다는 것을 의미한다. GSK3 $\beta$ 의 knock-down은 TuJ1<sup>+</sup> 세포의 수를 증가시킨 반면, GSK3 $\alpha$ 의 knock-down은 아무런 영향을 주지 않았다. GSK3 $\beta$  (Y216F)를 과발현시켰을 경우에는 신경세포 분화의 억제 효과가 나타나지 않았는데 이는 GSK3 $\beta$ 의 kinase 활성 자체가 그 역할에 중요하다는 것을 나타낸다. 초음파 영상을 이용하여 E9.5 생쥐의 뇌에 GSK3 $\beta$  발현 레트로바이러스 벡터를 전달한 *in vivo*

실험에서도 이와 유사한 결과가 관찰되었다. 또한, SB216763 를 처리하면 rapamycin 처리에 의해 억제된 신경세포 분화가 다시 회복되는 것이 관찰되었다. 이러한 결과는 (GSK3  $\alpha$  가 아니라) GSK3 $\beta$ 만이 신경세포 분화를 억제하며, 이는 mTORC1 신호 전달 경로의 하위 단계에서 작용하는 것을 의미한다.

IFN- $\gamma$ 는 바이러스 감염 시에 분비되는 대표적인 사이토카인 중 하나이다. 숙주 면역 반응에서 IFN- $\gamma$ 가 중요하다는 것은 잘 알려졌다으나 뇌 발달 측면에서 수행된 많은 *in vitro* 연구들은 상반되는 결과를 보였고, *in vivo* 실험은 전혀 없었다. E14.5 의 NPCs 에 IFN- $\gamma$ 를 처리하였을 경우, TuJ1<sup>+</sup> 신경 세포의 비율이 현저히 감소하였고, Nestin<sup>+</sup> NPCs 의 비율이 크게 증가하였다. 초음파 영상을 이용하여 E9.5 생쥐의 뇌에 IFN- $\gamma$  발현 레트로바이러스 벡터를 전달한 *in vivo* 실험에서도 유사한 결과가 나타났다. JAK 억제제인 Ruxolitinib 을 처리하거나 STAT1 의 발현을 knock-down 시켰을 경우에는 IFN- $\gamma$ 로 인한 신경세포 분화의 억제가 사라지는 것이 관찰되었다. 흥미롭게도 IFN- $\gamma$  처리를 한 NPCs 에서는 전신경 유전자 (proneural gene)의 하나인 *Neurogenin2* (*Neurog2*)의 발현이 억제되었으며, *Neurog2*를 과발현하는 세포에서는 IFN- $\gamma$ 의 효과가 나타나지 않았다. IFN- $\gamma$ 처리와 항상 활성을 가지도록 조작된 STAT1 돌연변이 단백질의 과발현은 *Neurog2* 프로모터의 활성을 약 절반으로 감소시켰다. 이 결과는 IFN- $\gamma$ 가 JAK/STAT1 신호전달 체계를 통해 *Neurog2*의 발현을 프로모터 수준에서 부분적으로 조절함으로써 NPCs 의 신경세포 분화를 억제한다는 사실을 의미한다.

이 연구를 통해 GSK3 $\beta$ 는 mTORC1 신호체계의 하위단계에서, IFN- $\gamma$ 는 JAK/STAT1 신호체계의 상위단계에 작용하여 신경세포 분화를 강하게 억제한다는 사실을 밝혔다. 이를 통해 신경세포 분화관점에서 GSK3 두 개 동형단백질의 역할을 최초로 명확히 밝혔으며, IFN- $\gamma$ 의 효과를 *in vivo* 에서 처음으로 확인하고, 하위 기전을

제시하였다. 이 논문의 연구 결과는 여러 스트레스 관련 인자들이 초기 뇌 발생에 미치는 영향과 작용 메커니즘을 이해하는데 기여할 수 있을 것으로 생각된다.

**핵심어:** 신경발생, 신경줄기세포 분화, 태아기 스트레스, Nrf2, GSK3, IFN- $\gamma$ , SC4MOL



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이학박사학위논문

**Embryonic Neurogenesis 에서  
스트레스 관련 인자들의 역할  
Roles of Stress-related Factors  
in Embryonic Neurogenesis**

2015년 12월

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## ABSTRACT

Stress exposure during development can lead to severe neurological diseases in infants and children. Many genes are known to be related to stress induction, but their roles in brain development have not been clearly elucidated. To rapidly screen for biologically meaningful factors involved in brain development, I first selected several genes that are known to be up-regulated in the fetal brain during prevalent stressed conditions such as prenatal infection and alcohol exposure, including nuclear factor (erythroid-derived 2)-like 2 (Nrf2), glycogen synthase kinase 3 (GSK3)  $\alpha$ , GSK3 $\beta$ , interferon-gamma (IFN- $\gamma$ ), and sterol-C4-methyl oxidase-like gene (SC4MOL). These genes were overexpressed in primary embryonic day (E) 14.5 murine neural progenitor cells (NPCs) using retroviral vectors. The percentage of neuronal cells was measured by TuJ1 expression. Using this method, GSK3 $\beta$  and IFN- $\gamma$  were found to have strong negative effects on neurogenesis, and they were further characterized *in vitro* and *in vivo* using various molecular techniques.

GSK3 is known as an important regulator during the proliferation and differentiation of NPCs, but the roles of the isoforms of this molecule (GSK3 $\alpha$  and GSK3 $\beta$ ) have not been clearly defined. Thus, the functions of GSK3 $\alpha$  and GSK3 $\beta$  in the context of neuronal differentiation of NPCs were characterized. Treatment of primary NPCs with a GSK3 inhibitor (SB216763) resulted in an increase in the percentage of TuJ1-positive immature neurons, suggesting an inhibitory role of GSK3 in embryonic neurogenesis. Downregulation of GSK3 $\beta$  expression increased the percentage of TuJ1-positive cells, whereas the knock-down of GSK3 $\alpha$  appeared to have no effect. Mutant GSK3 $\beta$  (Y216F) failed to suppress neuronal differentiation, indicating that the kinase activity of GSK3 $\beta$  is important for this regulatory function. Similar results were obtained *in vivo* when a retroviral vector expressing GSK3 $\beta$  was delivered to E9.5

mouse brains. In addition, SB216763 was found to block the rapamycin-mediated inhibition of neuronal differentiation of NPCs. Taken together, these data demonstrate that GSK3 $\beta$ , but not GSK3 $\alpha$ , negatively controls the neuronal differentiation of NPCs and that GSK3 $\beta$  may act downstream of the mTORC1 signaling pathway.

IFN- $\gamma$  is one of the critical cytokines released by host immune cells upon infection. Despite the important role(s) of IFN- $\gamma$  in host immune responses, there have been no *in vivo* studies of the effects of IFN- $\gamma$  on brain development, and the results obtained from many *in vitro* studies have been controversial. Treatment of E14.5 murine NPCs with IFN- $\gamma$  resulted in a decrease in the percentage of TuJ1-positive immature neurons but an increase in the percentage of Nestin-positive NPCs. Similar results were obtained *in vivo*. Treatment of NPCs with a JAK inhibitor or the knock-down of STAT1 expression abrogated the IFN- $\gamma$ -mediated inhibition of neurogenesis. Interestingly, the expression of one of proneural genes, *Neurogenin2* (*Neurog2*) was inhibited dramatically upon IFN- $\gamma$  treatment, and cells overexpressing *Neurog2* did not respond to IFN- $\gamma$ . Both IFN- $\gamma$  treatment and overexpression of the constitutively active form of STAT1 reduced the *Neurog2* promoter activity by nearly half. These results suggest that IFN- $\gamma$  inhibits the neuronal differentiation of NPCs by negatively regulating the expression of *Neurog2* partially at the promoter level via the JAK/STAT1 pathway.

In this thesis work, I identified GSK3 $\beta$  and IFN- $\gamma$  as negative controllers of neuronal differentiation, which act downstream of the mTORC1 signaling pathway and upstream of the JAK/STAT1 pathway, respectively. This is the first study to clearly distinguish the roles of GSK3 isoforms in the context of neuronal differentiation and to investigate the effects of IFN- $\gamma$  on embryonic neurogenesis *in vivo*. The findings from this thesis may provide insights into the mechanism of action of different stress-related factors during the early period of brain development, especially the initiation of

neurogenesis and the possible consequences of congenital stress exposure.

**Keywords:** Embryonic Neurogenesis, NPC differentiation, Prenatal Stress, Nrf2, GSK3, IFN- $\gamma$ , SC4MOL



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## ABBREVIATIONS

APC	Adenomatous polyposis coli
APP	Amyloid precursor protein
ARE	Antioxidant response element
AS-C	Achaete-scute complex
Ato	Atonal
CBF1	C-promoter binding factor 1
CK1 $\alpha$	Casein kinase 1 $\alpha$
CNC-bZip	Cap 'n' collar type of basic region leucine zipper factor
DMEM	Dulbecco's modified Eagle's medium
Dsh	Dishevelled
E	Embryonic day
eGFP	Enhanced GFP
FASDs	Fetal alcohol spectrum disorders
Fz	Frizzled
GAS	IFN- $\gamma$ activating sequence
GFAP	Glial fibrillary acid protein
GOI	Gene of interest
GSK3	Glycogen synthase kinase
H&E	Hematoxylin and eosin
Hes	Hairy and enhancer of split paralogues
HO-1	Heme oxygenase-1
Id	Inhibitor of differentiation
IF	Immunofluorescence
IFN- $\gamma$	Interferon-gamma
IHC	Immunohistochemistry
INPs	Intermediate neurogenic progenitor cells

KO	Knock-out
LRP	Lipoprotein receptor-related protein
MAPKAP-K1	MAPK-activated protein kinase 1
Mash	Mammalian achaete-scute homologue
MIA	Maternal immune activation
MSCV	Murine stem cell virus
mTORC1	Mammalian target of rapamycin complex 1
Neurog2	<i>Neurogenin 2</i>
Neurogs	Neurogenins
NICD	Notch intracellular domain
NPCs	Neural progenitor cells
Nqo1	NAD(P)H quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid derived 2-related factor 2
Olig	Oligodendrocyte lineage transcription factor
Pax6	Paired box 6
PBS	Phosphate buffered saline
PC	Positive control
PKB	Protein kinase B
PP	Protein phosphatase
Ptc	Patched 1 receptor
ROR	Receptor tyrosine kinase-like orphan receptor
ROS	Reactive oxygen species
RT	Room temperature
Ryk	Receptor-like tyrosine kinase
SC4MOL	Sterol-C4-methyl oxidase-like gene
SD	Standard deviation
Shh	Sonic hedgehog
Smo	Smoothed



STAT1ca	Constitutively active form of STAT1
SVZ	Subventricular zone
TCF/LEF	T-cell factor/lymphoid enhancing factor
TSC	Tuberous sclerosis complex
UIGD	Ultrasound image-guided gene delivery
UTR	Untranslated region
VZ	Ventricular zone

# **CHAPTER I**

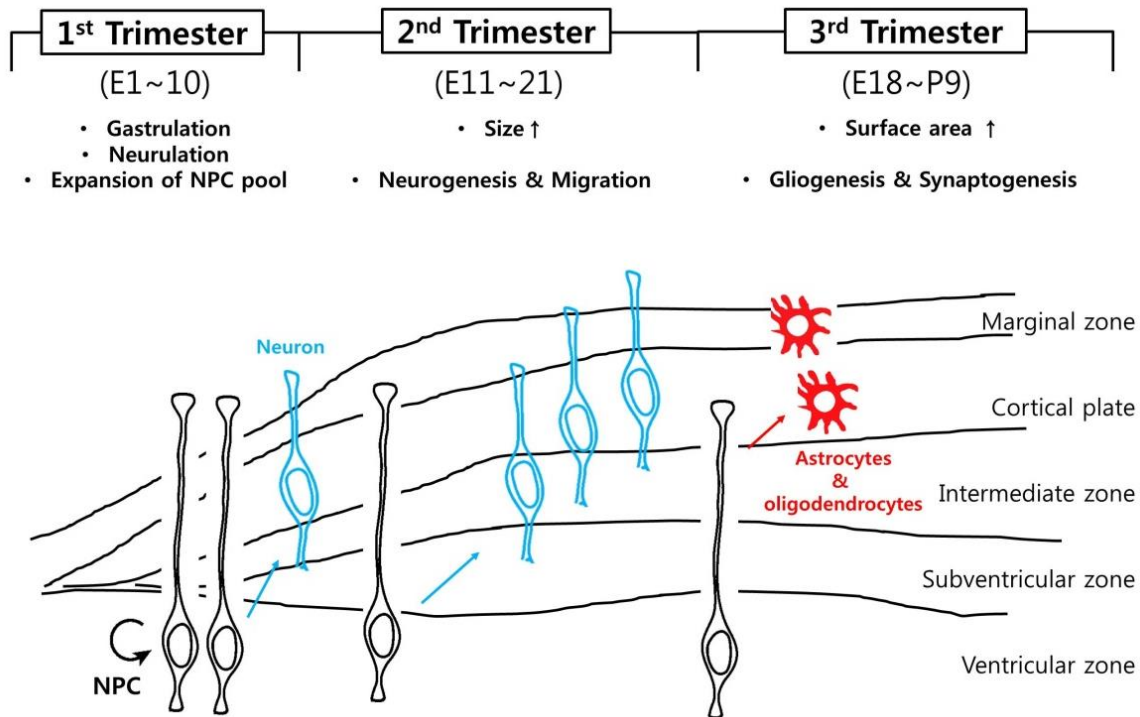
## **Introduction**

During brain development, various cellular pathways regulate numerous developmental processes including the proliferation, differentiation, and migration of cells, which occur concurrently in a spatiotemporal manner. Radical dynamic changes occur during a relatively short period of time; therefore, slight variations in genetic or environmental factors can cause severe developmental abnormalities. The degree and type of many congenital disorders vary greatly, and the underlying causes are unknown in more than 50% of the cases [1]. The main causes are believed to be environmental factors, i.e., stresses to which the mother or fetus have been exposed to such as smoking, radiation, lack of nutrients, and medication misuse. Among the many environmental causes, the two most prevalent stress conditions in our daily lives are prenatal alcohol exposure and congenital infection, which are associated with the increased risk of many neurological defects in newborn children such as microcephaly, delayed development, mental retardation, schizophrenia, and autism [2-5]. The identification of the biological processes responsible for these defects is critical yet difficult because a complete scientific understanding of embryonic brain development is lacking. As the first step to understand the relationship between environmental stress and neurodevelopment, I screened several stress-related genes to determine their involvement in neuronal differentiation. Factors with clear effects were selected, and their characteristics and roles in the differentiation of neural progenitor cells (NPCs) were further investigated *in vivo* as well as *in vitro*.

## **1. Brain development**

### **1.1 Overview of brain development**

Upon fusion of the sperm and egg, a zygote is formed, and it develops into a single entity through multiple steps over the course of 9 months. This developmental process can be divided into three 3-month-long trimesters (Fig. I-1). The brain starts to form in the 1<sup>st</sup> trimester and continues to develop even after birth. During the 1<sup>st</sup> trimester, the main structures of the brain appear and different parts begin to take shape. The brain grows continually during the 2<sup>nd</sup> trimester, and the major and



**Figure I-1. Overview of brain development.** During the 1<sup>st</sup> trimester, the main structures of the brain start to take shape through gastrulation and neurulation. NPCs undergo symmetric division to increase the size of the NPC pool at the VZ. Between the end of the 1<sup>st</sup> trimester and the beginning of the 2<sup>nd</sup> trimester, neuronal differentiation of NPCs is initiated, and the newly formed neurons start to migrate toward the cortical plate. In the 3<sup>rd</sup> trimester, gliogenesis and synaptogenesis start to occur. During this period, the size and surface area of the brain increase dramatically.

minor grooves are then formed in the 3<sup>rd</sup> trimester, increasing the surface area of the brain [6]. During this period, dramatic morphological changes as well as numerous cellular processes occur [7].

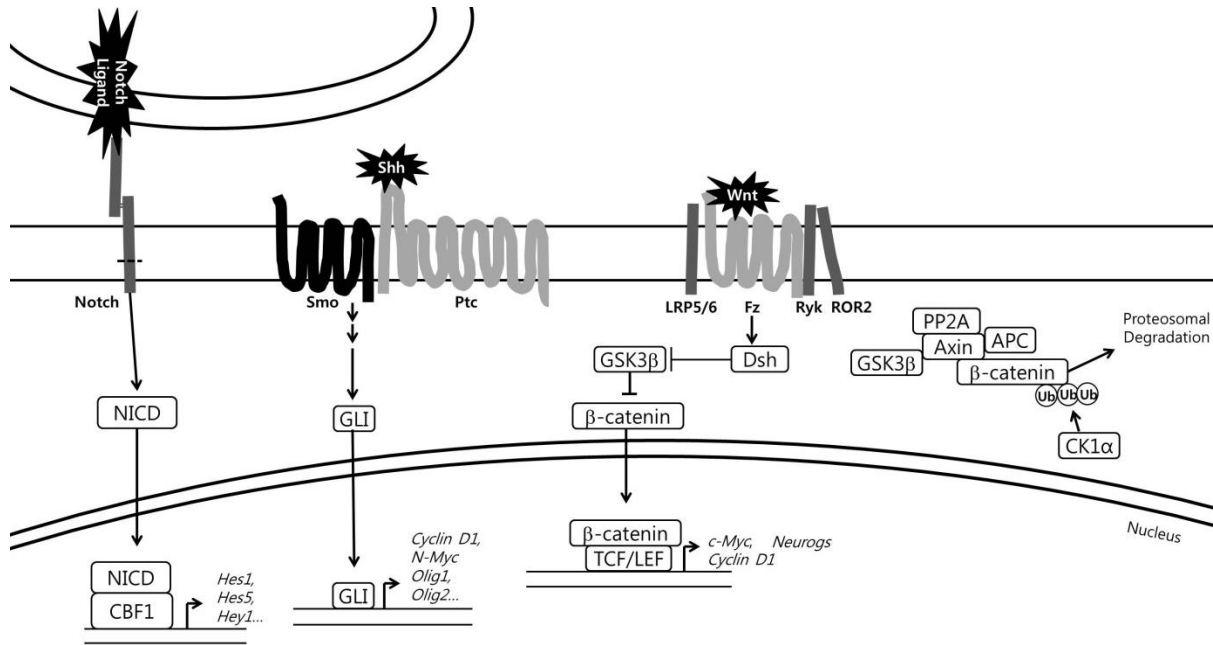
During the 1<sup>st</sup> trimester, NPCs, which can differentiate into different types of neural cells (neurons, astrocytes, and oligodendrocytes), actively proliferate in the ventricular zone (VZ) and increase the size of the NPC pool. In mice, NPCs start to differentiate into neurons at around embryonic day (E) 9. Differentiated neurons then begin to migrate from the VZ towards the cortical plate [8, 9]. In the 3<sup>rd</sup> trimester, synaptogenesis (the formation of synapses between neurons) begins and astrocytes and oligodendrocytes also appear around this time [10, 11]. In mice, the 3<sup>rd</sup> trimester corresponds to the postnatal period; therefore, this process takes place after birth. Brain development and NPC differentiation are executed through the activation, deactivation, and interaction of multiple signaling pathways [12, 13]. The transition to the next step requires various factors; however, the exact mechanism for each factor has not been clearly identified.

## **1.2 Major signaling pathways in NPCs**

A few crucial signaling pathways with major roles in the differentiation of NPCs are illustrated in Fig. I-2 and described in the following.

### **Notch**

Notch is a 300-kDa transmembrane receptor, which exists as a heterodimer and responds to Notch ligand proteins including Delta 1, 3, and 4, or Jagged 1 and 2. The role of Notch in brain development has been studied extensively, and factors that play roles in the Notch signaling pathway has been found to be highly expressed in NPCs [14, 15]. After the Notch ligand interacts with the extracellular domain of Notch, gamma-secretase cleaves the intracellular domain of Notch, releasing the Notch intracellular domain (NICD). NICD then translocates to the nucleus and interacts with the DNA binding protein C-promoter binding factor 1 (CBF1), also known as RBPJ or CSL, which allows the transcription of target genes [16]. Notch enhances the proliferation of NPCs and the differentiation



**Figure I-2. Major signaling pathways in NPCs.** Schematic diagrams of the Notch, Shh, and Wnt signaling pathways are shown. When Notch signaling is activated, NICD is released and translocates to the nucleus. NICD then associates with CBF1 and activates its downstream target genes. In the Shh pathway, activated Smo releases Gli, which initiates the transcription of downstream target genes. When the Wnt pathway is activated, Fz activates Dsh, which blocks the proteosomal degradation of β-catenin. β-catenin then associates with TCF/LEF and starts the transcription of its downstream target genes. NICD, Notch intracellular domain; CBF1, C-promoter binding factor 1; Smo, Smoothened; Ptc, Patched 1 receptor; Ci, cubitus interruptus; CtBP LRP5/6, lipoprotein receptor-related protein 5/6; Fz, Frizzled; Ryk, receptor-like tyrosine kinase; ROR2, receptor tyrosine kinase-like orphan receptor 2; Dsh, disheveled; TCF/LEF, T-cell factor/lymphoid enhancing factor; PP2A, protein phosphatase 2A; APC, adenomatous polyposis coli; CK1α, casein kinase 1α.

of astrocytes, but it inhibits neuronal differentiation by increasing the expression of genes in the hairy and enhancer of split paralogues (Hes) family, such as *Hes1* and *Hes5* [17, 18].

### **Sonic hedgehog**

Sonic hedgehog (Shh) is a well-known morphogen that belongs to the Hedgehog family and it is an essential factor in numerous developmental processes including segmentation, organogenesis, and cell differentiation. Shh binds to the membrane-bound Patched 1 receptor (Ptc) and releases the inhibitory activity of Ptc on a membrane embedded protein Smoothed (Smo). Activation of Smo then initiates the nuclear translocation of Gli, which acts as a transcription factor. In the context of NPC differentiation, Gli activates the genes that regulate NPC proliferation and oligodendrocyte differentiation such as cyclin D1, *N-Myc*, and oligodendrocyte lineage transcription factor 1 and 2 (*Olig1* and *Olig2*) [19, 20].

### **Wnt**

Wnt is a secreted glycoprotein that binds to the N-terminal extracellular domain of the transmembrane receptor Frizzled (Fz). Fz interacts with other co-receptors (lipoprotein receptor-related protein 5/6, LRP5/6; receptor-like tyrosine kinase, Ryk; and receptor tyrosine kinase-like orphan receptor 2, ROR2), and binding to Wnt activates the cytoplasmic protein dishevelled (Dsh), which transduces the signal to downstream targets. In the canonical Wnt pathway (Wnt/ $\beta$ -catenin pathway),  $\beta$ -catenin plays a major role as a transcription factor.  $\beta$ -catenin normally forms a destruction complex with Axin, adenomatous polyposis coli (APC), protein phosphatase 2A (PP2A), and GSK3. In the absence of Wnt, Casein kinase 1 $\alpha$  (CK1 $\alpha$ ) ubiquitinates  $\beta$ -catenin and leads to its proteosomal degradation. However, when Wnt signaling is activated, activated Dsh inhibits GSK3 in the destruction complex, allowing the release of  $\beta$ -catenin into the cytoplasm [21]. The accumulated  $\beta$ -catenin proteins translocate to the nucleus and associate with the T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factors, which regulate downstream target genes. Wnt plays various

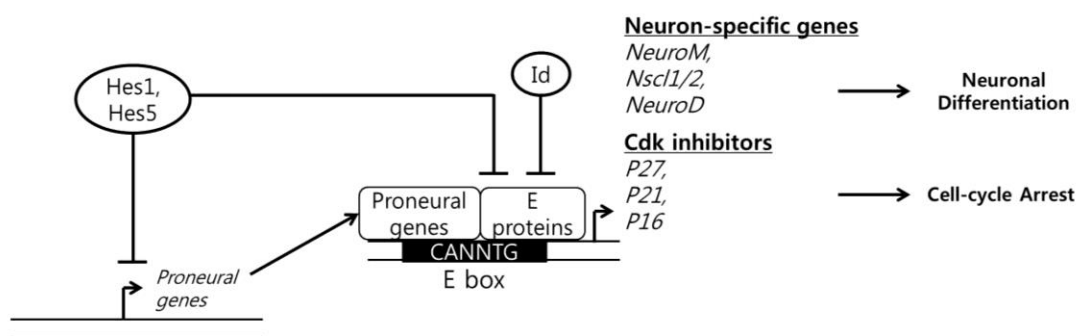
roles in development including body axis formation, cell proliferation, and differentiation. In terms of neurogenesis, it induces the proliferation of early NPCs by regulating cell cycle-related genes, such as *c-myc* and *cyclin D1*, and in the later stage of neurogenesis (E13.5), it induces proneural genes such as *neurogenins* (*Neurogs*) [22-31].

### 1.3 Proneural genes

Downstream target genes of the aforementioned signaling pathways determine the differentiation of NPCs by regulating the expression of proneural genes, which are essential for normal neural differentiation [32, 33]. Proneural genes encode proteins with a characteristic molecular structure, which comprises two alpha helices connected with a short loop. These are called the basic helix-loop-helix (bHLH) transcription factors and most function as transcriptional activators. bHLH factors form homo- or heterodimers with other bHLH factors such as E proteins, and bind to the E-box (CANNTG) consensus sequence (Fig. I-3). The expression of bHLH factors in self-renewing neuroepithelial cells induces commitment to a neuronal fate instead of a glial fate [33]. When these factors are activated, they upregulate the expression of Notch ligands such as *Delta* or *Jagged*, which bind to Notch receptor in neighboring cells, inhibiting their neuronal differentiation.

Proneural genes are divided into two main groups based on their sequence similarity: genes of the achaete-scute complex (AS-C) including the *mammalian achaete-scute homologue 1* and 2 (*Mash1/2*), and the atonal (Ato) group including *Neurog 1*, 2, and *D*, and *Math1* [34]. The deletion of proneural genes delays or impairs neuronal development and causes abnormal cell proliferation or brain morphology. The two main genes that are required and sufficient for the neurogenic program are *Mash1* and *Neurog2*, which are expressed in a non-overlapping pattern. *Mash1* is expressed in the progenitor cells of the ventral telencephalon and olfactory epithelium, whereas *Neurog2* is expressed in the progenitor cells of the dorsal telencephalon and ventral spinal cord. *Mash1* and *Neurog2* are responsible for the generation of different neuronal types (autonomic GABAergic neurons, olfactory receptor neurons, and dopaminergic neurons).





**Figure I-3. Proneural gene pathway.** When proneural genes are expressed, they form homo- or heterodimers with E proteins, and bind to the E-box (CANNTG) consensus sequence. This then activates the expression of neuron-specific genes and Cdk inhibitors, leading to neuronal differentiation and cell-cycle arrest, respectively. Hes1 and Hes5 inhibit this process by directly binding to the promoter of proneural genes, blocking their expression, or by interacting with E proteins, inhibiting the activity of heterodimers. Inhibitor of differentiation (Id) also blocks dimer formation of proneural factors.

Neurogenesis occurs in a specific spatiotemporal manner, so the activity of proneural genes is tightly regulated by various mechanisms. *Inhibitor of differentiation (Id)* genes inhibit the activity of proneural genes by binding to E proteins with a high affinity and form heterodimers that cannot bind to DNA [35, 36]. *Hes* genes block the activity of proneural genes by repressing their transcription or by interfering with complex formation of proneural genes and E-protein [32, 37-39]. At the onset of neurogenesis, different proneural genes are expressed in a sequential manner leading to a process known as the bHLH cascade. Early expression of bHLH transcription factors regulates the late expression of other bHLH genes, for example, paired box 6 (Pax6) activates *Neurog2* expression, which leads to the expression of *Tbr2* and *NeuroD* in intermediate neurogenic progenitor cells (INPs), which result in the induction of *Tbr1* expression in INPs and early differentiating neurons [40, 41].

## 2. Commonly used techniques in brain research

As described above, several factors have been investigated in the context of brain development. However, the exact mechanisms underlying the regulation of each factor or the cross-regulation between different pathways have not been clearly investigated, and different studies have often suggested contradictory results. Among many possible explanations, one cause might be due to the technical drawbacks associated with the currently available brain research models.

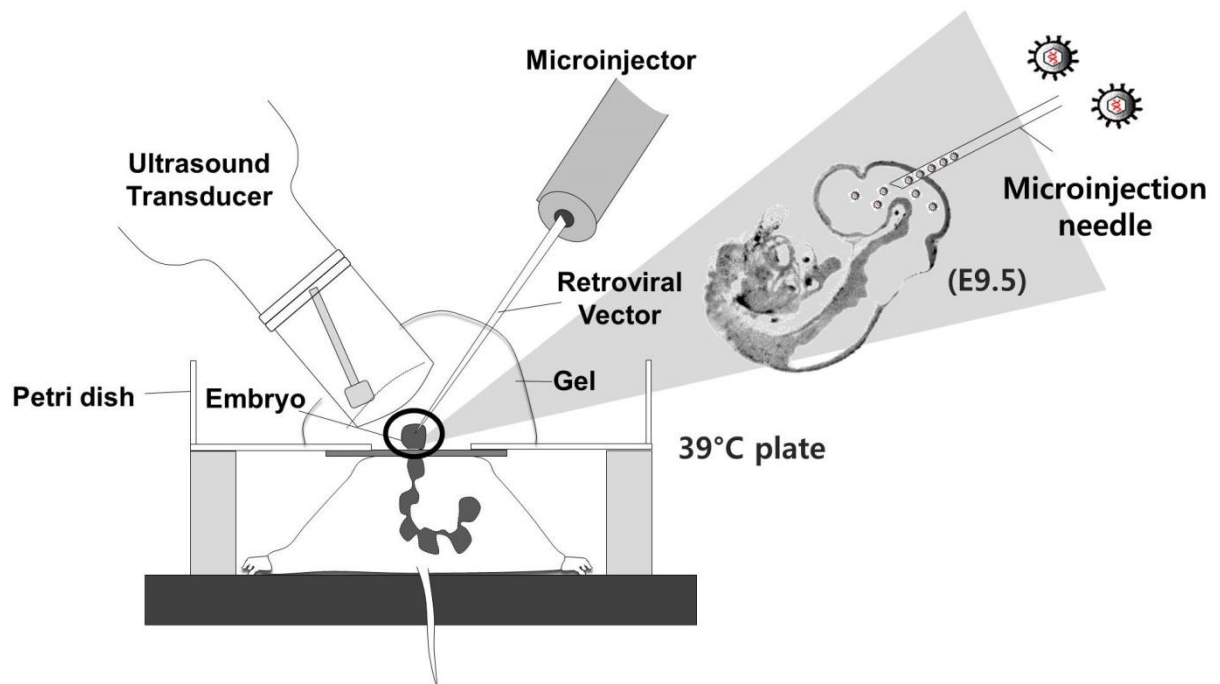
Most *in vivo* studies investigating the effects of various factors on embryonic brain development use mouse models with germline modifications such as transgenic or knock-out (KO) mice. However, this method is not perfectly suitable for studying the specific roles of factors because genetic manipulations affect all cell types and organs in animals, which may cause premature death [42-44]. One can manipulate genes only in a specific type of cell using a cell-specific promoter, but the expression level of the transgene can change and similar genes can compensate for the gene loss over time [45, 46]. Furthermore, when screening of multiple genes is necessary, germline modification is not the best method to proceed as it requires tremendous effort over a long period of time and high maintenance costs.

Another commonly used technique for studying the effects of genes on brain development is *in utero* electroporation, which requires the injection of a plasmid that expresses the gene of interest (GOI) directly into the embryonic brain. However, this method does not permit investigation of the early stages of cell differentiation because the embryo needs to be sufficiently large to enable the identification of the injection target site. Therefore, experiments cannot be performed before E13-14, although neuronal differentiation has already begun by this developmental stage [47].

The ultrasound image-guided gene delivery (UIGD) technique can overcome the limitations of the abovementioned methods [48, 49]. The embryonic brain contains an empty cavity called the forebrain ventricle. While the embryo is monitored by ultrasound imaging, a viral vector expressing the GOI can be injected into this ventricle using a microinjection pipette (Fig. I-4). Gene transfer at the early stage has numerous advantages compared with transfer at E13, which is when *in utero* electroporation is usually performed. Neurogenesis begins at E9–10 [47], and therefore, the early developmental effects of a specific gene can be determined. In addition, more cells can be transduced with the same amount of virus and the expression of the transgene can be maintained by using a retrovirus, which integrates its genome into cellular chromosomes. Most importantly, the UIGD technique is a quick and cost-effective method for producing animals carrying a cerebral transgene, avoiding the compensation effect and alterations in the gene expression level. Given these advantages, the UIGD technique was utilized in the present study to deliver a retrovirus expressing the GOI at E9.5.

### **3. Overview of thesis research**

In this thesis, five stress-related factors were selected for the reasons described in Chapter III, and initially screened to determine their effects on embryonic neurogenesis. Their roles in neuronal differentiation were quickly screened *in vitro* using primary NPCs and confirmed *in vivo*. GSK3 $\beta$  and IFN- $\gamma$  were selected on the basis of their clear negative effects on the neuronal differentiation of NPCs, and further characterizations were performed through various *in vitro* experiments. The results of this



**Figure I-4. Overall procedure of the ultrasound image-guided gene delivery (UIGD) technique.**

The uterus of anesthetized pregnant mice is exteriorized and the fetuses are scanned using an ultrasound biomicroscopic imaging system. A viral vector expressing the GOI is injected into the telencephalic ventricle of E9.5 embryos using ultrasound-guided imaging. The embryos are then transferred back to their original site and grown until analyzed. (Taken from Jang *et al.*, 2013 [50])

study are believed to facilitate the development of preventive drugs or treatment against neurodevelopmental diseases, and may help to understand the crucial steps of neurodevelopment.

## **CHAPTER II**

### **Materials & Methods**

## 1. Cell culture

293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco). NIH3T3 cells were cultured in DMEM supplemented with 10% bovine serum (Gibco). All cells were incubated in 5% CO<sub>2</sub>, 37°C chamber.

## 2. Isolation and *in vitro* culture of mouse neural progenitor cells

All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University. The ganglionic eminences in E14.5 embryonic brains were dissected and washed twice in phosphate buffered saline (PBS). Samples were triturated using a fire-polished Pasteur pipette, and run through a 40-µm cell strainer (Falcon). Cells were cultured in NPC proliferating media, which contains DMEM/F12 (Gibco) with B27 supplement (2% (v/v), 17504-044, Gibco), N2 supplement (1% (v/v), 17502-048, Gibco), Pen Strep Glutamine (1% (v/v), 10378-016, Gibco), 2 µg mL<sup>-1</sup> heparin (H3393, Sigma), 20 ng mL<sup>-1</sup> hFGF basic (100-18B, Pepro Tech), and 10 ng mL<sup>-1</sup> EGF (AF-100-15, Pepro Tech) and incubated in 5% CO<sub>2</sub>, 37°C chamber.

After one day, neurospheres were dissociated into single cells and transduced with retroviral vectors as described below. Two days later, neurospheres were dissociated and seeded on poly-L-ornithine (0.01%, P4957, Sigma) and laminin (10 µg mL<sup>-1</sup>, L2020, Sigma) -coated plates. The cells were incubated in DMEM containing 2% FBS (Gibco) with or without rapamycin (9904, Cell Signaling) and/or SB216763 (S3442, Sigma) dissolved in DMSO. The final concentrations of DMSO were 0.02% (v/v) for rapamycin and 0.01% (v/v) for SB216763.

In experiments with IFN-γ, cells were cultured in new proliferating media for 2 days and differentiated with DMEM containing 2% FBS (Gibco) with or without recombinant mouse IFN-γ (IF005, Millipore), Ruxolitinib (S1378, Selleck) or cycloheximide (Sigma) on poly-L-ornithine (Sigma) and laminin (Sigma)-coated plates.

### 3. Plasmid constructs

The MS vector containing the long terminal repeat from a murine stem cell virus (MSCV) was described previously [51].

The murine GSK3 $\alpha$ , Nrf2, and *Neurog2* promoter without splicing site (#8) were synthesized (Bioneer, Korea). The murine GSK3 $\beta$ , IFN- $\gamma$ , SC4MOL, Neurog2, STAT1, and *Neurog2* promoter sequences [14] were amplified from cDNAs isolated from Raw 264.7, the PMA/Ionomycin stimulated mouse splenocytes, NIH3T3, adult murine hippocampal tissue, HT22 cells, and genomic DNA isolated from murine macrophage cell line (Raw 264.7), respectively using primers listed in Table II-1. The murine NICD sequence was amplified from the plasmid called GNIA [52] as a template. These sequences were cloned into pGEM $\text{\textcircled{R}}$  -T Easy (A1360, Promega) according to the manufacturer's instruction. To construct the mutant GSK3 $\beta$  (Y216F), mutant *Neurog2* promoters, and constitutively active STAT1 (STAT1ca) sequences, site-directed mutagenesis (Stratagene) was employed using pGEM $\text{\textcircled{R}}$  -T Easy-GSK3 $\beta$ , -*Neurog2* promoter, and -STAT1 as a template, respectively. Mutant plasmids were confirmed by sequencing. Verified sequences were cloned into the MS-IRES-eGFP, pGL3-Basic (E1751, Promega), or pGL4.17 [luc2/Neo] (E6721, Promega) vector.

The shRNA sequences (Table II-2) targeting murine GSK3 $\alpha$ , GSK3 $\beta$ , STAT1 or luciferase (as the control) were cloned into pSIREN-DsRed (Clontech) vector, which was manipulated by site-directed mutagenesis to contain HA tag. The shRNA sequence targeting luciferase was used as the control.

### 4. Retroviral vectors and transduction procedure

Retroviral vector plasmids were transfected into 293T cells with gag-pol (pCA-gag-pol) and env-expressing vectors (pCA-VSV-G) using Lipofectamine (18324-012, Invitrogen) and PLUS reagent (10964-021, Invitrogen), according to the manufacturer's instructions. Supernatants were



Gene	Primer sequence
Nrf2	F 5'-GGATCC GCCACC ATGATGGATCTGGAGCTGCCCCCTC-3' R 5'-AGATCT TCAATTTTTCTTAGTGTCGGGTT-3'
SC4MOL	F 5'-ACGCGTATGGCCACAAACAAAAGTGTT-3' R 5'-GGATCCTCAATCAGACTTTTTCCCAAG-3'
GSK3 $\beta$ (Y216F)	F 5'-GGAGAGCCCAATGTTTCATTATCTGTTCTCGGTACTAC-3' R 5'-GTAGTACCGAGAACAGATAAATGAAACATTGGGCTCTCC-3'
GSK3 $\beta$	F 5'-AGATCTGCCACCATGTCGGGGCGACCGAGA-3' R 5'-AGATCTTCAGGAAGAGCTAGCGAG-3'
NICD	F 5'-GGATCCGCCACCATGGCTCAGCATGGCCAGCTCTG-3' R 5'-GGATCCCTAAGCGTAATCTGGAACATCGTATGGGTAGCTCGAGCTGTCCAACAGG-3'
IFN- $\gamma$	F 5'-GGATCC GCCACC ATGAACGCTACACACTGCAT-3' R 5'-AGATCT TCAGCAGCGACTCCTTT-3'
Neurog2	F 5'-ACGCGTGCCACCATGTTTCGTCAAATCTGAGACT-3' R 5'-AGATCTCTAGATACAGTCCCTGGCGAG-3'
STAT1	F 5'-ACGCGTGCCACCATGTCACAGTGGTTCGAGCT-3' R 5'-AGATCTTTATACTGTGCTCATCACTGTCAA-3'
STAT1ca	F 5'-AACTACAAAGTCATGGCTTGTGAGTGTATACCAGAGAATCCCCTG-3' R 5'-CAGGGGATTCTCTGGTATACACTCACAAGCCATGACTTTGTAGTT-3'
Neurog2 promoter #1	F 5'-CCCCTAATGAGCTGCTGAAAGG-3' R 5'-CCATGGATCCTGAGAAAGAAGAGGCAGATG-3'
#2	F 5'-GAGCTCACTGATTGGCCAGCTGCGCCTC-3' R 5'-CCATGGATCCTGAGAAAGAAGAGGCAGATG-3'
#3	F 5'-GAGCTCTCCAGCCGAGCGCGAGCCACCAAT-3' R 5'-CCATGGATCCTGAGAAAGAAGAGGCAGATG-3'
#4	F 5'-GAGCTCGGAGAGGGACTAAAGAAAGGGA-3' R 5'-CCATGGATCCTGAGAAAGAAGAGGCAGATG-3'
#5	F 5'-GAGCTCTTTTCTTGGTGGTATATAAGGGGTT-3' R 5'-CCATGGATCCTGAGAAAGAAGAGGCAGATG-3'
#6	F 5'-CCCCTAATGAGCTGCTGAAAGG-3' R 5'-CCATGGCCTACGCGGCTCCAGATG-3'
#7	F 5'-CCCCTAATGAGCTGCTGAAAGG-3' R 5'-CCATGGCGCCCGAGTCTCGTGTGT-3'

**Table II-1. PCR and mutagenesis primers sequences**

<b>Gene</b>	<b>shRNA sequence</b>
GSK3 $\alpha$ #1	5'-GAAGTGGCTTACACTGACA-3'
GSK3 $\alpha$ #2	5'-GAAGGTTCTTCAGGACAAA-3'
GSK3 $\alpha$ #3	5'-GCTGGACCACTGCAATATTGT-3'
GSK3 $\beta$ #1	5'-GCATGAAAGTTAGCAGAGA-3'
GSK3 $\beta$ #2	5'-GAAAGTTAGCAGAGATAAA-3'
GSK3 $\beta$ #3	5'-GAAAGTGATTGGAAATGGA-3'
STAT1 #1	5'-GGATCAAGTCATGTGCATA-3'
STAT1 #2	5'-TTGCAAGAGCTGAACTATA-3'
STAT1 #3	5'-GCCGAGAACATACCAGAGAAT-3'
Luciferase	5'-GTGCGTTGCTAGTACCAAC-3'

**Table II-2. shRNA sequences**

collected 48 hr after transfection, filtered through a 0.45- $\mu$ m filter and frozen at -80°C until used. Concentrated viral stocks were prepared by ultracentrifugation for 2 hr in a SW32 rotor (Beckman-Coulter) at 20,000 r.p.m. at 4°C. Pellets were resuspended in 50  $\mu$ l of PBS at 4°C overnight, and aliquots of virus were stored at -80°C. For transduction, NIH3T3 cells were seeded at  $1 \times 10^5$  in six-well plates on the previous day. Viral supernatants were added in the presence of polybrene (final concentration 8  $\mu$ g mL<sup>-1</sup>). In the case of mouse primary NPCs, ultracentrifuge-concentrated virus particles were added in the absence of polybrene and incubated at 37°C for 3 hr. After incubation, the cells were washed and cultured in the NPC medium as described above. The viral titer was determined by measuring the percentage of eGFP, dsRED or HA-positive NIH3T3 cells transduced with different dilutions of virus stock. When FACS analysis was needed, the percentages of eGFP<sup>+</sup> cells were analyzed using *FACS Calibur* flow cytometer (BD Biosciences).

## 5. Western blot

NIH3T3 cells or primary NPCs were lysed using RIPA buffer or CytoBuster™ Protein Extraction Reagent (Novagen) with protease and phosphatase inhibitor cocktail. Equal amounts of protein were resolved by 10% (w/v) SDS-PAGE and transferred to PVDF membranes (GE Healthcare). The membranes were blocked with TBST (150 mM NaCl, 10 mM Tris/HCl, 0.1% (v/v) Tween 20, pH 8.0) containing 1% (w/v) BSA (Invitrogen-Gibco) or 5% (w/v) skim milk, and incubated with primary antibodies (listed in Table II-3) diluted in blocking solution at 4°C overnight. The membranes were then incubated with HRP-conjugated goat anti-mouse or rabbit IgG secondary antibody (Sigma) at room temperature (RT) for 1 hr. The bands were visualized with the enhanced chemiluminescence system (WBKLS0500, Millipore), and quantified using Image J software. When needed, blots were stripped using stripping buffer (NCI1059KR, Thermo Scientific) with vigorous shaking for 30 min at RT.

<b>Antibody</b>	<b>Catalogue #</b>	<b>Company</b>	<b>Dilution (IF/IHC/WB)</b>
Rabbit anti-GFP	A11122	Invitrogen	1:500 (IF/IHC)
Rat anti-HA tag	11-867-423-001	Roche	1:300 (IF)
Mouse anti-class III $\beta$ -tubulin (TuJ1)	MMS-435P	Covance	1:500 (IF/IHC)
Mouse anti-Nestin	MAB353	Millipore	1:300 (IF)
Mouse anti-GFAP	MAB3402	Millipore	1:300 (IF/IHC)
Mouse anti-NeuN	MAB377	Millipore	1:50 (IHC)
Rabbit anti-GSK3 $\alpha$	ab28833	Abcam	1:250 (IF)
Mouse anti-GSK3 $\alpha$	sc166116	Santa Cruz	1:100 (WB)
Mouse anti-GSK3 $\beta$	Ab93926	Abcam	1:300 (IF), 1:000(WB)
Alexa Fluor® 488-donkey anti-rabbit IgG	A21206	Invitrogen	1:1000 (IF) 1:500 (IHC)
Alexa Fluor® 488 Donkey Anti-Mouse IgG Antibody	A21202	Invitrogen	1:1000 (IF)
Alexa Fluor® 555-donkey anti-mouse IgG	A31570	Invitrogen	1:1000 (IF) 1:500 (IHC)
Alexa Fluor® 555 Goat Anti-Rat IgG	A21434	Invitrogen	1:1000 (IF)
Alexa Fluor® 488 Goat Anti-Rat IgG	A11006	Invitrogen	1:1000 (IF), 1:500 (IHC)
Hoechst 33258	09460	Polyscience	1:5000 (IF)
Mouse anti-Neurog2	MAB3314	R&D systems	1:100 (WB)
Rabbit anti-total STAT1	9172S	Cell Signaling	1:500 (WB)
Rabbit anti-P-STAT (Tyr701)	9167	Cell Signaling	1:500 (WB)
Mouse anti-phospho-GSK3 (pY216)	612313	BD Bioscience	1:1000 (WB)
Rabbit anti-phospho-GSK3 $\alpha/\beta$ (Ser21/9)	9331	Cell Signaling	1:500 (WB)
Rabbit anti-phospho-p70S6K (Thr389)	9205	Cell Signaling	1:1000 (WB)
Rabbit anti-p70S6K	2708	Cell Signaling	1:1000 (WB)
Mouse anti- $\beta$ -actin	A-5441	Sigma	1:5000 (WB)

**Table II-3. Antibodies used for IF, IHC and Western blot (WB)**

## **6. Quantitative RT-PCR**

Total RNA was isolated using RNAiso Plus (Takara). 1 $\mu$ g of each RNA was used to synthesize cDNAs using Reverse Transcriptase XL (AMV) (Takara) according to the manufacturer's instruction. Quantitative PCR was performed using SYBR Premix Ex Taq (Takara) and the primers listed in Table II-4.

## **7. *In vivo* injection into the ventricle of the embryonic brain**

All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University. Timed pregnant ICR mice (Orient-Bio or Koatech, Korea) were used for viral injections. Virus delivery was performed into the telencephalic ventricle at E9.5 using the ultrasound-guided imaging as described previously [53]. Briefly, pregnant mice were anesthetized with Zoletil 50 (Virbac) and rompun (Bayer Korea). The uterus was exteriorized, and the fetuses were scanned using the ultrasound biomicroscopic imaging system (Vevo660; VisualSonics). Ultracentrifuge-concentrated virus particles were injected into the telencephalic ventricle of E9.5 embryos. Embryos were transferred back into the womb and allowed to grow until E14.5.

## **8. Brain harvest and fixation**

E14.5 brains from embryos were harvested and fixed in 4% paraformaldehyde for 2 hr at 4°C and cryoprotected with 30% sucrose at 4°C until they dropped to bottom. Brains were then frozen in embedding matrix (Cell Path) and stored at -80°C until they were cryosectioned.

## **9. Immunofluorescence, immunohistochemistry assays, and H&E staining**

Immunofluorescence (IF) and immunohistochemistry (IHC) assays were performed using the primary and secondary antibodies listed in Table 3. For IF, cells were fixed with 4%

Gene	Primer sequence
<i>Neurog2</i>	F 5'–AACTCCACGTCCCCATACAG–3' R 5'–GAGGCGCATAACGATGCTTC–3'
<i>Math1</i>	F 5'–GTAAGGAGAAGCGGCTGTG–3' R 5'–AGCCAAGCTCGTCCACTA–3'
<i>Mash1</i>	F 5'–GCAACCGGGTCAAGTTGGT–3' R 5'–GTCGTTGGAGTAGTTGGGGG–3'
<i>Tbr2</i>	F 5'–GGCCCCTATGGCTCAAATTC–3' R 5'–CCTGCCCTGTTTGGTGATG–3'
<i>IRF1</i>	F 5'–AGAGGAACCAGAGATTGACAGC–3' R 5'–GTCAGAGACCCAAACTATGGTGC–3'
<i>SC4MOL</i>	F 5'–AAACAAAAGTGTTGGCGTGTTTC–3' R 5'–AAGCATTCTTAAAGGGCTCCTG–3'
<i>GAPDH</i>	F 5'–CTGGAAAGCTGTGGCGTGAT–3' R 5'–CCAGGCGGCACGTCAGATCC–3'

**Table II-4. qRT-PCR primer sequences**

paraformaldehyde, permeabilized with PBS containing 0.5% Triton X-100, and then blocked for 1 hr with PBS containing 10% FBS. The samples were incubated first with primary antibodies diluted in blocking solution overnight at 4°C, and then incubated with secondary antibodies diluted in PBS for 1 hr at room temperature. Hoechst staining was done with 1  $\mu\text{g mL}^{-1}$  Hoechst 33258 (09460, polyscience). For IHC assay, sections were washed in PBS, and then blocked for 1 hr with PBS containing 1% FBS and 0.2% Triton X-100. Sections were incubated first with primary antibodies diluted in blocking solution overnight at 4°C, and then incubated with secondary antibodies diluted in blocking solution for 1 hr at room temperature. Hoechst staining was done with 1  $\mu\text{g mL}^{-1}$  Hoechst 33342. Hematoxylin and eosin (H&E) staining was done by Histopathology Core Lab in Seoul National University (Seoul, Korea).

The stained cells or sections were visualized and their images were acquired using Zeiss (Oberkochen, Germany) LSM 510 or LSM700 confocal microscope. Cells were counted using Image J software. Signal intensity was quantified using ZEN 2012 (blue edition) software.

## **10. Migration assay**

E14.5 primary NPCs were transduced with the control or GSK3 $\beta$ -expressing retroviral vectors. After 2 days, eGFP-positive cells were sorted using FACS Aria III (Becton Dickinson).  $1.25 \times 10^5$  of sorted cells suspended in 100  $\mu\text{L}$  of F12 DMEM were plated on PLO and laminin coated 8.0  $\mu\text{m}$  polycarbonate membrane. The lower chamber contained 600  $\mu\text{L}$  of 2% FBS in F12 DMEM with 100 ng/mL SCF (255-sc, R&D systems). After 1 day, the upper surface of the membrane was washed, and the membrane was fixed with 4% PFA for 30 min at RT and stained with crystal violet for 30 min at RT.

E14.5 primary NPCs were allowed to form neurospheres and were allowed to differentiate with or without IFN- $\gamma$  for 1 day. Cells were fixed, permeabilized, blocked and stained with TuJ1-specific antibody as described above. The distance from the edge of the neurosphere to the furthest

outgrowing cell was measured and normalized to the diameter of the neurosphere using Image J software.

## **11. TUNEL assay**

Cells were fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.5% Triton X-100, and then blocked for 1 hr with PBS containing 10% FBS. TUNEL staining was performed using the in situ Cell Death Detection Kit TMR red (Roche), according to the manufacturer's instructions. The stained cells were visualized and their images were acquired using Zeiss (Oberkochen, Germany) LSM 510 or LSM 700 confocal microscope. Cells were counted using Image J software.

## **12. MTT assay**

NPCs were differentiated with various concentrations of IFN- $\gamma$  for 2 days. Cell viability was analyzed using MTT assay kit (Roche) according to the manufacturer's protocol.

## **13. Luciferase reporter assay**

pNeurog2-Luc or pNeurog2 $\Delta$ -Luc with p $\beta$ -gal were co-transfected into NIH3T3 cells using Lipofectamine and PLUS reagent (Invitrogen), according to the manufacturer's protocol. After 3 hr, fresh media was added with or without 50 ng/mL IFN- $\gamma$ . NPCs were transfected with pNeurog2-Luc or pNeurog $\Delta$ -Luc, using Neon<sup>®</sup> Transfection System (Invitrogen) (1500V, 10ms, 3pulses). After 1 day, cells were differentiated with or without 50 ng/mL IFN- $\gamma$  in DMEM containing 2% FBS for 12 hr. All cells were lysed with Reporter Lysis Buffer (Promega) and the firefly luciferase assay reagent was added to equal amounts of cell lysates. Transfection efficiency was measured using  $\beta$ -galactosidase assay. The luminescent signal was measured using a microplate luminometer.



For stable integration, Neurog2pro-Luc-Neo<sup>R</sup> was linearized with *Bam*HI. A total of  $1.6 \times 10^6$  NIH3T3 cells were suspended in 400  $\mu$ L of serum-free medium mixed with 10  $\mu$ g of linearized Neurog2pro-Luc-Neo<sup>R</sup> plasmid, and subjected to electroporation using Gene Pulser Xcell electroporation system (220V, 975  $\mu$ F, Bio-Rad) followed by G418 selection (25  $\mu$ g/ml). G418-resistant cell clone with the highest basal luciferase activity was selected, expanded, and subjected to luciferase reporter assay as described above.

## **14. Statistical analysis**

All values are presented as mean  $\pm$  standard deviation (SD) from three independent experiments. Differences between values were determined by a one-way ANOVA followed by Tukey's post-hoc test or two-way ANOVA, using GraphPad Prism software (Version 5, GraphPad Software). Differences in the signal intensity from IF assays and the band intensity from Western blot analysis were determined by unpaired t-test and paired t-test, respectively using GraphPad Prism software. P-values less than 0.05 were considered to be statistically significant.

## **CHAPTER III**

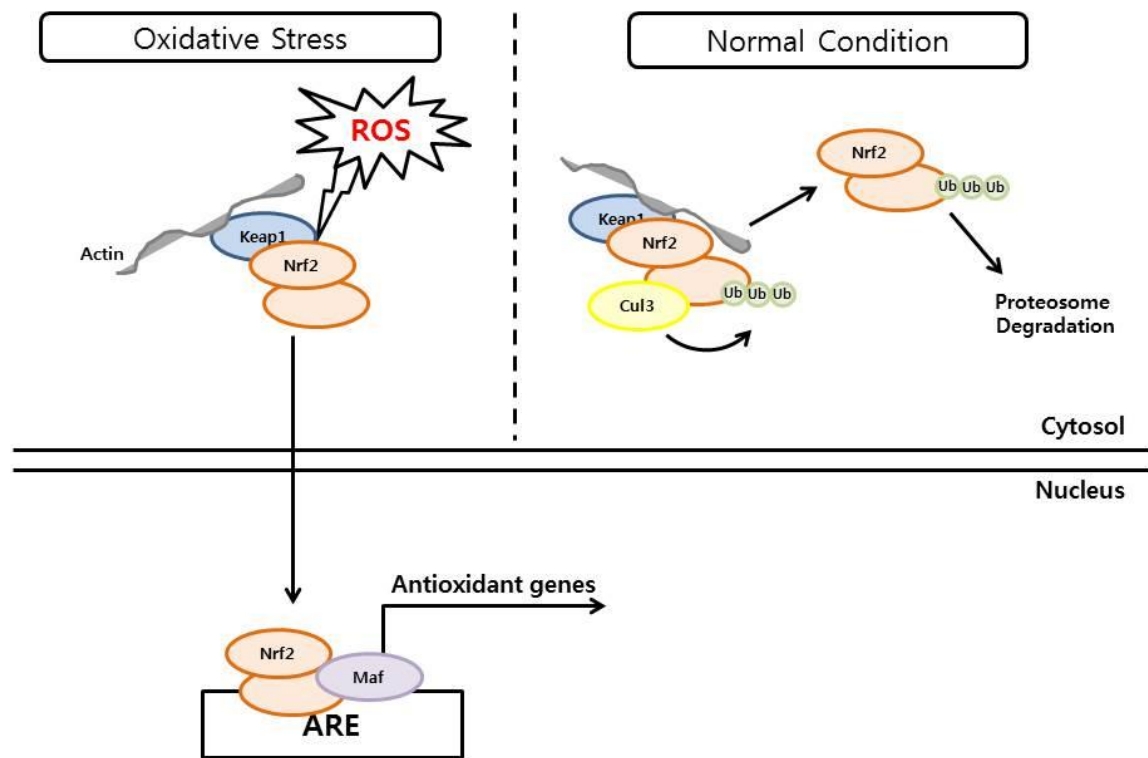
# **Effects of Stress-related Genes on Embryonic Neurogenesis**

# 1. Background

The most prevalent risk factors that can lead to serious brain defects are alcohol consumption and infection during pregnancy. Children of mothers who have been exposed to alcohol during pregnancy exhibit an increased risk of mental retardation, microcephaly, and functional brain problems such as memory loss and cognitive defects [2, 54-56]. Similarly, children who experienced an infection during their fetal development have a higher risk of various brain defects such as microcephaly, schizophrenia, autism, cerebral palsy, and mental retardation [57-62]. In this chapter, five candidate factors (Nrf2, GSK3 $\alpha$ , GSK3 $\beta$ , IFN- $\gamma$ , and SC4MOL) which have increased expression levels during fetal alcohol exposure or prenatal infection were selected, and their effects on the initiation of neurogenesis were investigated.

## 1.1 Rationale for choosing Nrf2

The Nrf2-antioxidant response element (ARE) pathway is a well-known pathway in the antioxidant system, which is activated upon oxidative stress such as alcohol exposure that generates reactive oxygen species (ROS) in the mother's womb and the fetus [63]. When oxidative stress occurs, transcription factor Nrf2 translocates to the nucleus and regulates the expression of numerous proteins that contribute to the antioxidant response such as heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (Nqo1), and glutamate-cysteine ligase (Fig.III-1). Alcohol consumption induces the generation of ROS in both the mother and fetus, which leads to an approximately three-fold increase in Nrf2 expression in the fetus [64, 65]. Despite its expression during embryonic development, the role of Nrf2 in fetal development has been elusive. Nrf2 KO mice exhibit no developmental or reproduction deficits; therefore, the role of Nrf2 in development has not been studied extensively. However, a study published in 2003 using a double KO mouse line for Nrf2 and Nrf1, both of which belong to the same cap 'n' collar type of basic region leucine zipper factor (CNC-bZip) family, suggested that members of the CNC-bZip family might compensate for each other as the double KO



**Figure III-1. Schematic representation of Nrf2 signaling pathway.** In normal conditions, Nrf2 is constitutively expressed and bound by actin-anchored protein Kelch-like ECH associating protein 1 (Keap1), which is an adaptor protein of Cullin-3-based E3 ubiquitin ligase complex (Cul3). Cul3 continuously ubiquitinates Nrf2 and leads to its proteosomal degradation. In the presence of oxidative stress, the redox-sensitive cysteine residue (C273 and 288) of Keap1 is oxidized, resulting in a conformational change and the release of Nrf2. The free Nrf2 then translocates into the nucleus and forms a heterodimer with Maf protein. This complex binds to ARE and regulates the expression of antioxidant genes.

mice have a shorter *in utero* life compared with the Nrf1 KO mice [66]. Based on this finding, several studies have investigated the roles of Nrf2 in cell differentiation and development; however, these are at an early stage [67, 68].

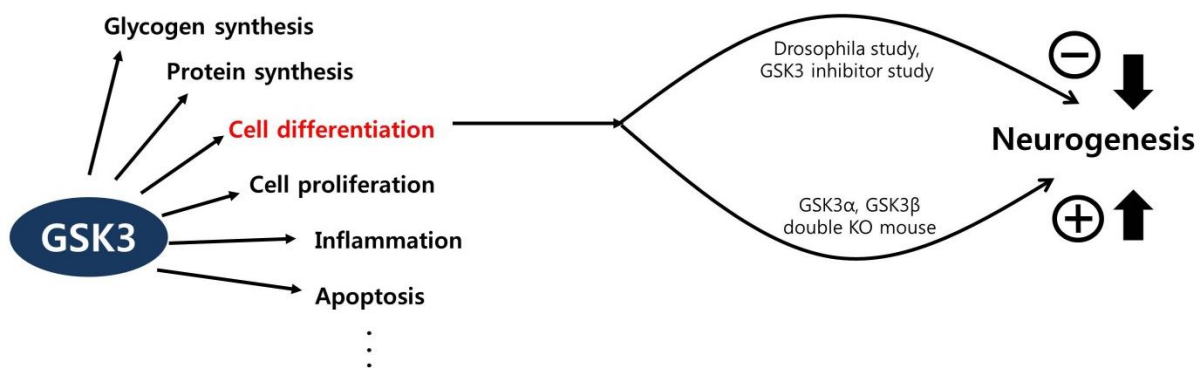
## 1.2 Rationale for choosing GSK3

GSK3 is a serine/threonine kinase, which is involved in numerous physiological processes including glucose metabolism, apoptosis, cellular proliferation, and differentiation. GSK3 has two isoforms, GSK3 $\alpha$  and GSK3 $\beta$ , which share 98% homology in their kinase domains. It is involved in cellular division and differentiation, but its role in the differentiation of NPCs is controversial (Fig. III-2). Several *in vitro* studies have been performed using chemical inhibitors that bind to the ATP binding pocket of GSK3 to block its kinase activity. Studies using these GSK3 inhibitors showed that GSK3 inhibits neurogenesis. However, chemical inhibitors cannot distinguish between the two isoforms of GSK3; therefore, the specific role of each isoform has not been determined. In addition, these experiments were conducted only *in vitro*.

*In vivo* investigation of the two isoforms began after GSK3 transgenic animals became available. In contrast to the results obtained using inhibitors, GSK3 $\alpha$  or GSK3 $\beta$  single KO mice do not show any significant cerebral defects. However, hyperproliferation and decreased differentiation of NPCs are detected in the brains of GSK3 $\alpha/\beta$  double KO mice. Therefore, the precise roles of the GSK3 isoforms in embryonic neurogenesis are unclear and controversial, and thus, functional investigation of GSK3 isoform is necessary.

## 1.3 Rationale for choosing IFN- $\gamma$

In infants or children, congenital viral infections can lead to severe neurological diseases such as blindness, epilepsy, hydrocephalus, and mental retardation; these in turn can increase the risk of schizophrenia and autism [57-62]. Fetal brain damage might be induced by the pathogen itself; however, it has been suggested that the activated immune response may be the main cause of this



**Figure III-2. Contradictory results regarding the role of GSK3 in NPC differentiation.** GSK3 is involved in various cellular processes, but the exact roles of each isoform are unclear. No major brain malformations have been found in GSK3 $\alpha$  KO mice or in mice engineered to have the GSK3 $\beta$  gene deleted in their NPCs, whereas the deletion of both the GSK3 $\alpha$  and GSK3 $\beta$  genes induces hyperproliferation and suppresses neuronal differentiation. By contrast, a *Drosophila* mutant with a defective *shaggy* gene, a GSK3 homologue, exhibits increased neuronal differentiation. Use of GSK3 inhibitors also promotes neuronal differentiation of human NPCs, rat ventral midbrain precursors, and rat neural stem cells.

pathogenesis [69]. IFN- $\gamma$  is a critical cytokine released by host immune cells upon infection, but despite its significant roles in the host immune response, the effects of IFN- $\gamma$  on fetal brain development has not been clearly understood.

Several studies have been performed to determine the function of IFN- $\gamma$  in NPC differentiation; however, different results were reported depending on the experimental conditions (Fig.III-3). In addition, the downstream mechanisms regulated by IFN- $\gamma$  have not been clarified, and there have been no *in vivo* results related to its function in the embryonic brain. In this thesis, the effects of IFN- $\gamma$  on brain development were investigated using both *in vitro* and *in vivo* experimental systems.

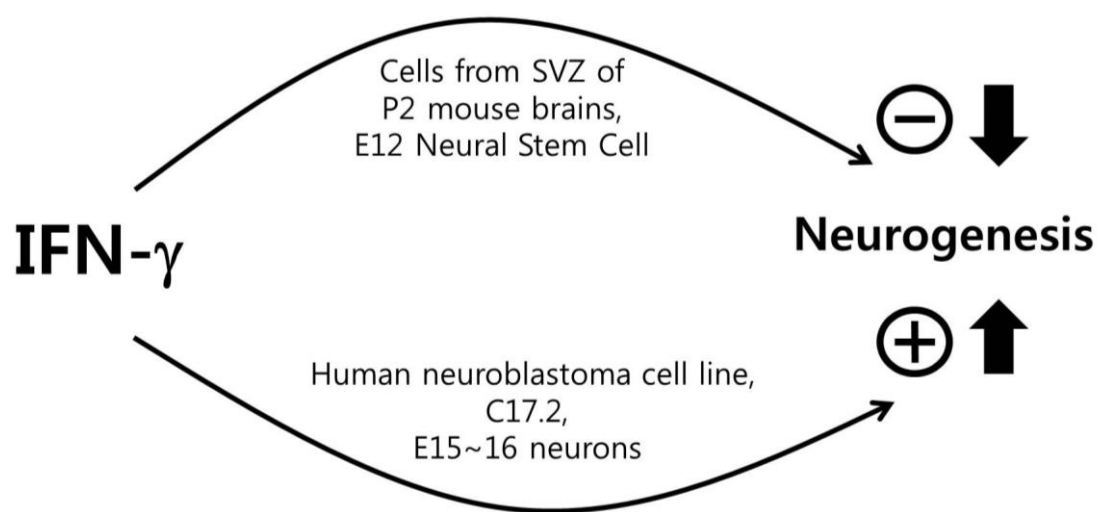
## **1.4 Rationale for choosing SC4MOL**

SC4MOL is involved in cholesterol synthesis. Defects in cholesterol synthesis can lead to severe symptoms such as developmental defects or neonatal lethality, but no study has addressed the relationship between SC4MOL and brain development. In 2011, a case report showed that a patient with psoriasiform dermatitis, arthralgia, congenital cataracts, microcephaly, and developmental delay possessed two point mutations in the SC4MOL gene (519T  $\rightarrow$  A and 731A  $\rightarrow$  G) [70]. A study published in 2012 reported that the expression level of SC4MOL was increased in the fetal brain in three maternal immune activation (MIA) models [71]. Based on these results, SC4MOL was chosen to investigate whether it has an effect on the differentiation of NPCs.

## **2. Results**

### **2.1 Strategy for studying candidate genes**

In order to investigate the role of each factor in the most efficient manner, an *in vitro* screening method was employed using mouse primary NPCs. To perform gain of function studies, retroviral vectors that express the GOI and the enhanced green fluorescent protein (eGFP) reporter



**Figure III-3. Contradictory results regarding the role of IFN- $\gamma$  in NPC differentiation.** IFN- $\gamma$  induced neurogenesis in experiments involving a human neuroblastoma cell line, neonatal NPC line (C17.2), and E15-E16 neurons. However, IFN- $\gamma$  obtained the opposite result with cells from the subventricular zone of postnatal day 2 mouse brains, and E12 neural stem cells. SVZ, subventricular zone.



gene in a bicistronic message were created. The expression and activity of each gene was tested, and E14.5 NPCs were then transduced with the respective viral vectors and differentiated for 3 days (Fig. III-4).

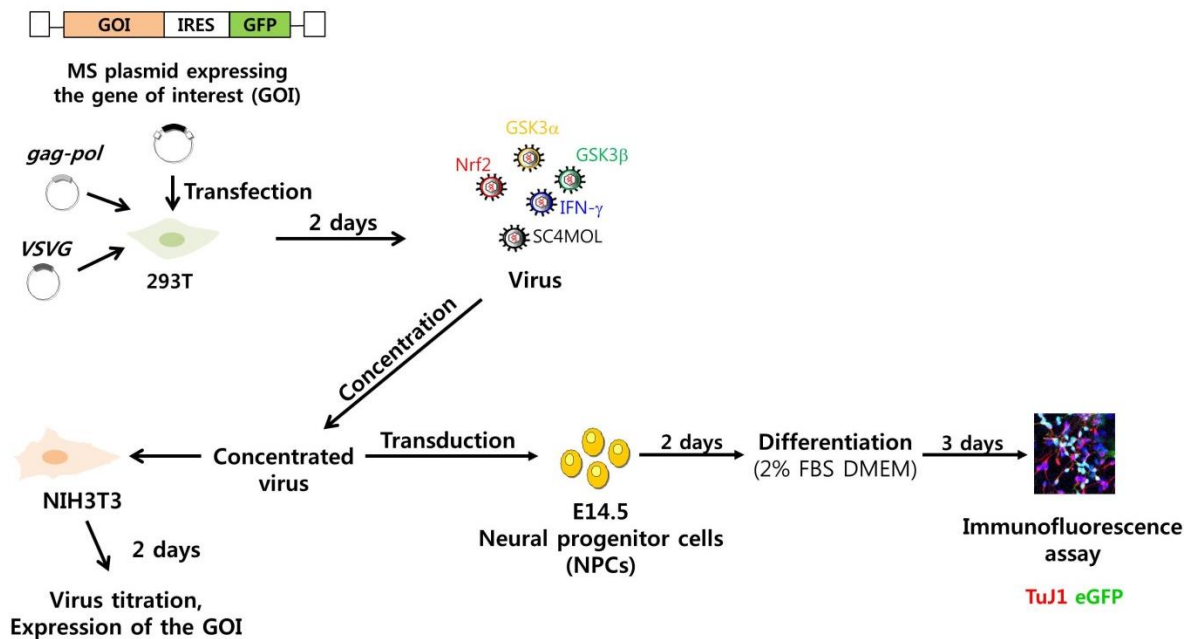
## **2.2 Effects of Nrf2 on embryonic neurogenesis**

To test the role of Nrf2 in NPC differentiation, a retroviral bicistronic vector that expressed both Nrf2 and eGFP was generated. The viral construct was transduced into NIH3T3 cells, and the expression of exogenous Nrf2 was determined by western blotting using an Nrf2-specific antibody (Fig. III-5A). Nrf2 was not detectable in the control cells, whereas it was detected in Nrf2-overexpressing cells. The activity of exogenous Nrf2 was determined by measuring the expression level of HO-1, a downstream target of Nrf2. As expected, the level of HO-1 increased by approximately four-fold in cells transduced with the Nrf2-expressing retroviral vector.

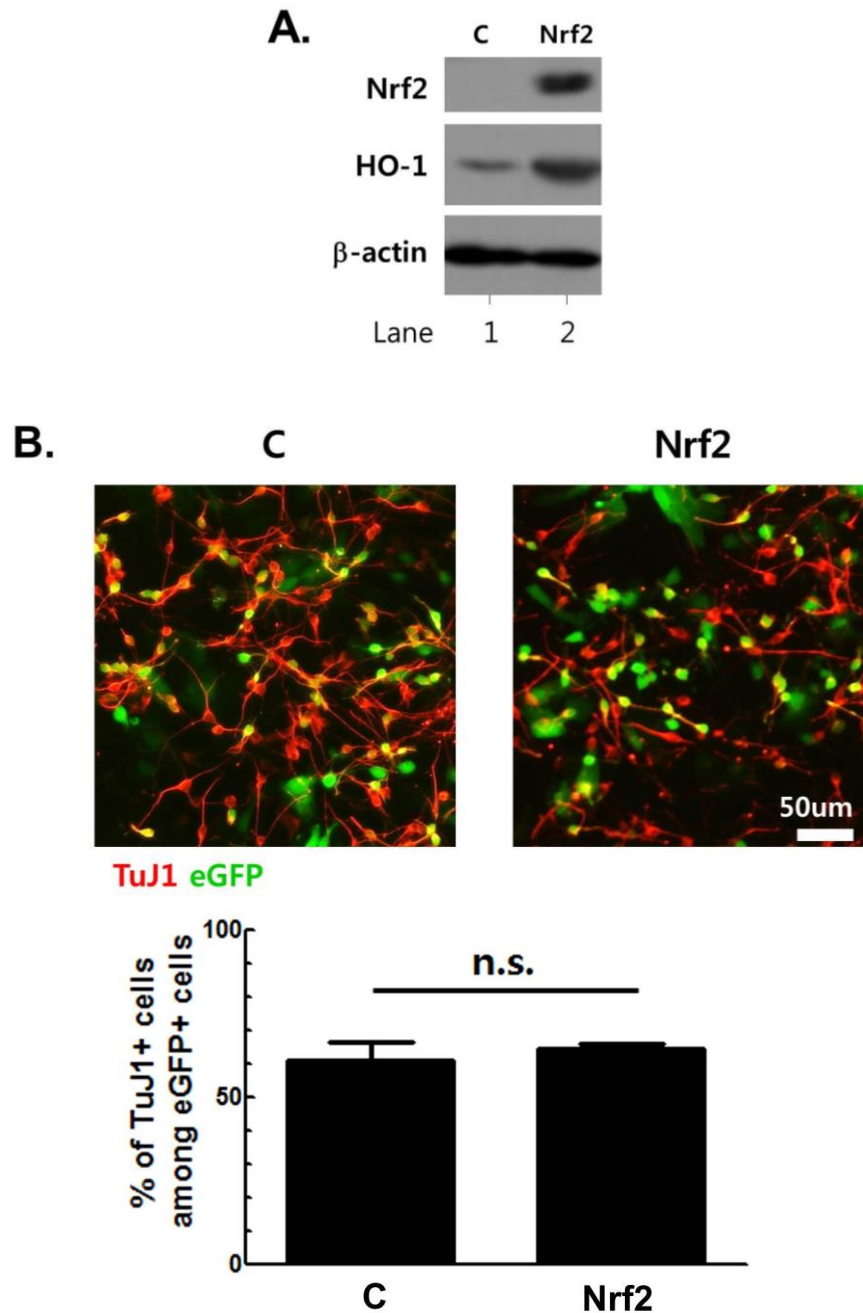
Primary NPCs were transduced with the retroviral vector expressing Nrf2, and differentiation was then induced for 3 days. Among the cells transduced with the control vector that only expresses eGFP, approximately 60% of the eGFP-positive cells were positive for the neuron-specific TuJ1. A similar ratio of TuJ1-positive cells was observed in the Nrf2-transduced cells, indicating that Nrf2 does not influence neuronal differentiation of NPCs (Fig. III-5B).

## **2.3 Effects of the GSK3 isoforms on embryonic neurogenesis**

To examine the roles of GSK3 isoforms in neuronal differentiation, the effect of GSK3 overexpression was investigated. NIH3T3 cells were transduced with retroviral vectors expressing GSK3 $\alpha$  or GSK3 $\beta$  together with eGFP, and the expression levels of the isoforms were determined using western blotting. Endogenous GSK3 $\alpha$  was not detectable, while endogenous GSK3 $\beta$  was visible only after longer exposures in the control cells. Exogenous GSK3 $\alpha$  and GSK3 $\beta$  were detected when



**Figure III-4. Overall procedure employed for *in vitro* screening of factors that affect neuronal differentiation of NPCs.** Retroviral vector plasmids expressing the GOI and eGFP in a bicistronic message were transfected into 293T cells with *gag-pol* (pCA-*gag-pol*) and *env*-expressing vectors (pCA-VSV-G). Supernatants were collected at 48 hr after transfection and concentrated by ultracentrifugation. The viral titre and the expression of the GOI were analyzed by transducing NIH3T3 cells with concentrated virus particles in the presence of polybrene. To determine the effects of each gene on the differentiation of NPCs, concentrated virus particles were added to E14.5 NPCs in the absence of polybrene. After 3 days, the cells were differentiated with DMEM containing 2% FBS and immunostained with antibodies specific to eGFP and neuron-specific TuJ1.



**Figure III-5. Effects of Nrf2 on the neuronal differentiation of NPCs.** (A) Retroviral vectors expressing Nrf2 together with eGFP were added to NIH3T3 cells for 2 days. The total cell lysates were subjected to western blotting using antibodies to Nrf2 and HO-1. (B) NPCs were transduced with the same titer of respective retroviral vectors and then induced to differentiate for 3 days, followed by immunostaining for eGFP with TuJ1. Representative images of the staining and the percentages of each cell type among the eGFP-positive cells are shown. Scale bar, 50  $\mu$ m. n.s., not significant; C, control.

cells were transduced with the respective retroviral vectors, and tyrosine phosphorylated GSK3, which is an indication of GSK3 activity, was also detected (Fig. III-6A).

Primary NPCs were transduced with retroviral vectors expressing GSK3 $\alpha$  or GSK3 $\beta$  at the same titer, and then differentiated for 3 days. Among the cells transduced with the control vector expressing eGFP only, approximately 60% of the eGFP-positive cells were positive for TuJ1. A similar ratio of TuJ1-positive cells was observed in GSK3 $\alpha$ -transduced cells, while this ratio was reduced to 20% in GSK3 $\beta$ -transduced cells (Fig.III-6B). These results indicate that GSK3 $\beta$ , but not GSK3 $\alpha$ , inhibits neuronal differentiation.

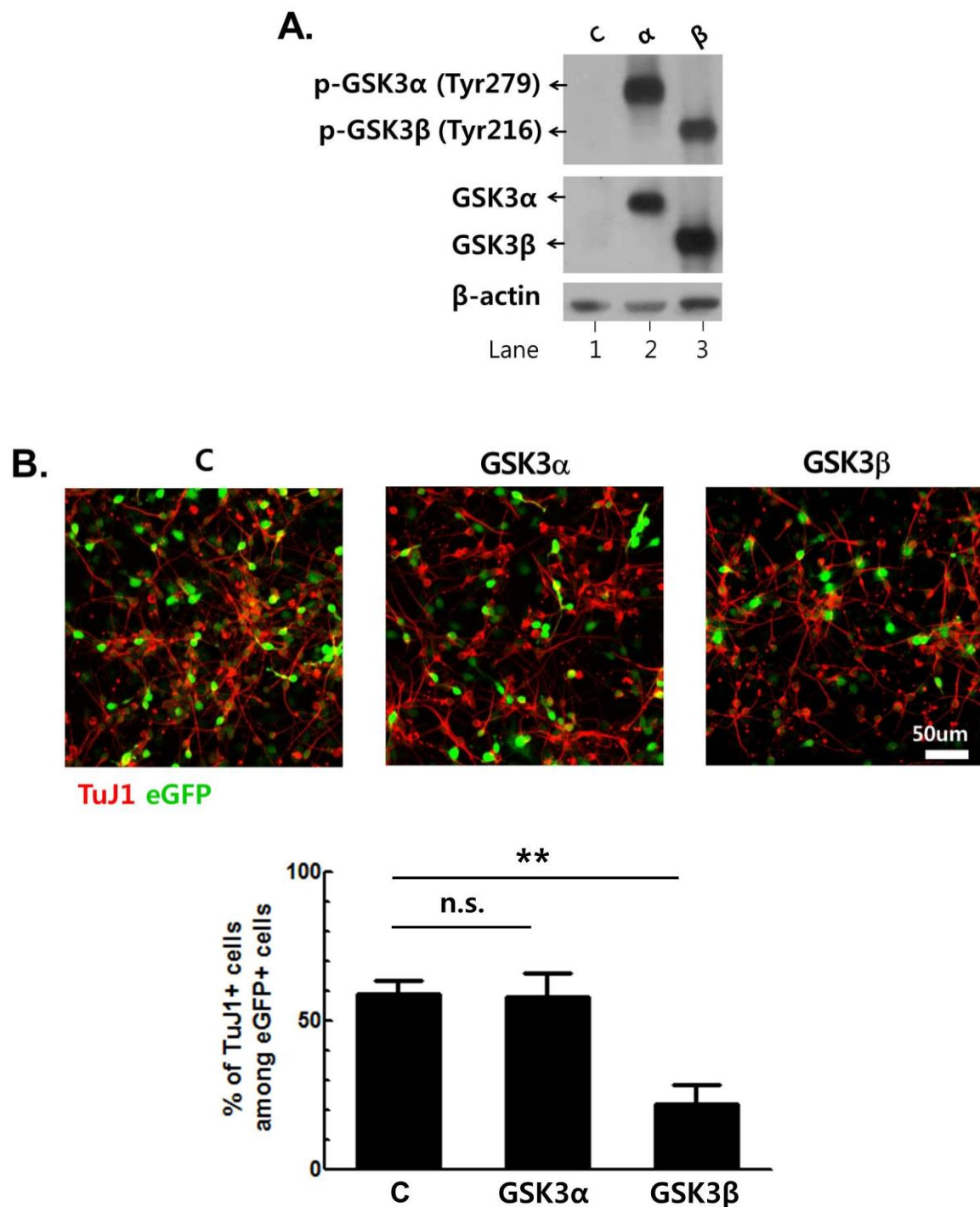
## **2.4 Effects of IFN- $\gamma$ on embryonic neurogenesis**

Similar to the aforementioned constructs, an IFN- $\gamma$ -expressing retroviral vector was generated and transduced into primary NPCs. IFN- $\gamma$  was not detected in the control cells, while approximately 10 ng/mL of IFN- $\gamma$  was secreted in the transduced NPCs (Fig. III-7A). Tyrosine-phosphorylated STAT1 was also detected, suggesting that the exogenous IFN- $\gamma$  is functional (Fig. III-7B).

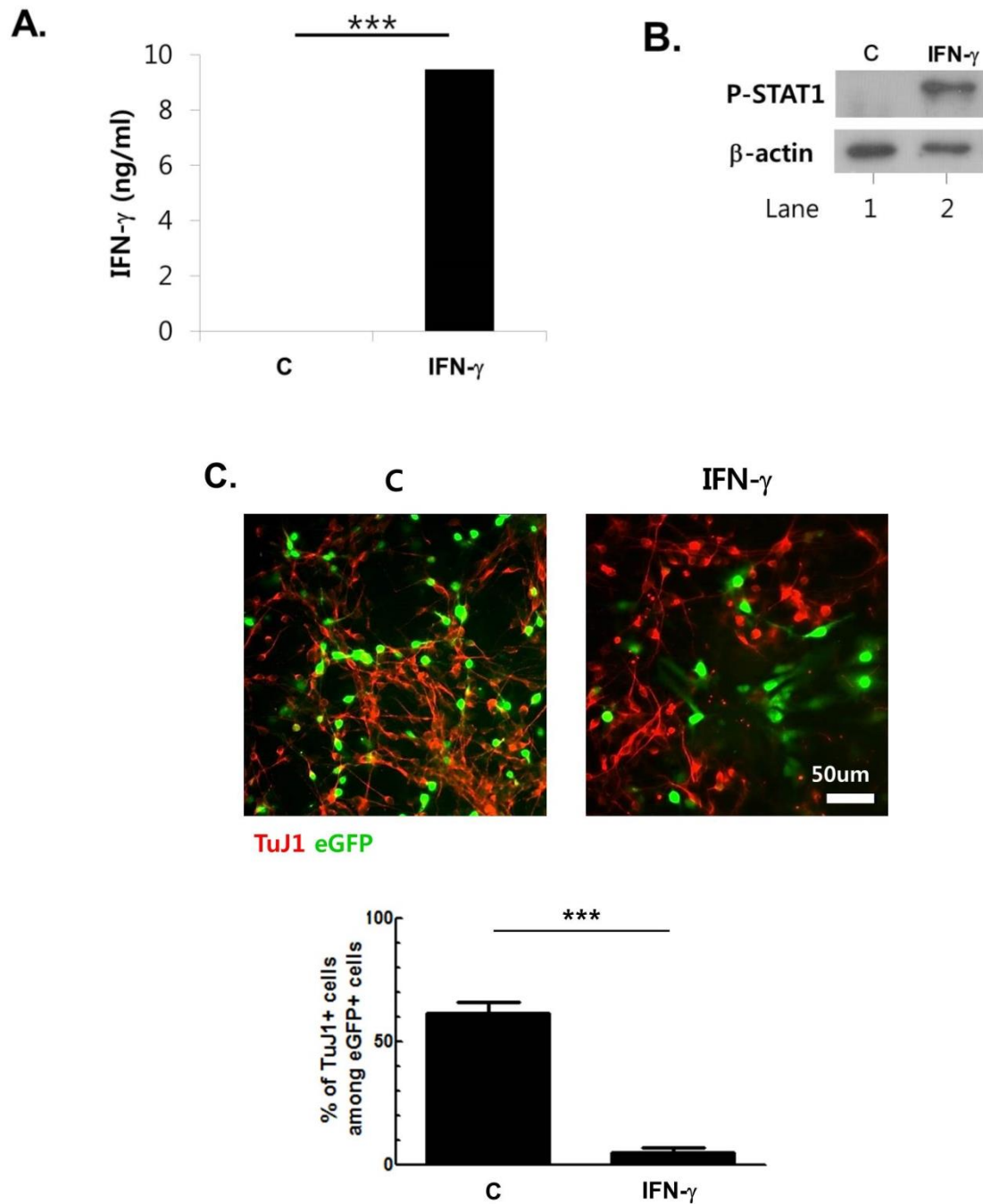
When primary NPCs were transduced with a retroviral vector that expresses IFN- $\gamma$  and differentiated, the ratio of TuJ1-positive cells dramatically decreased by approximately 70% compared with the cells transduced with the control construct (Fig. III-7C). This result demonstrates that IFN- $\gamma$  can strongly inhibit neuronal differentiation.

## **2.5 Effects of SC4MOL on embryonic neurogenesis**

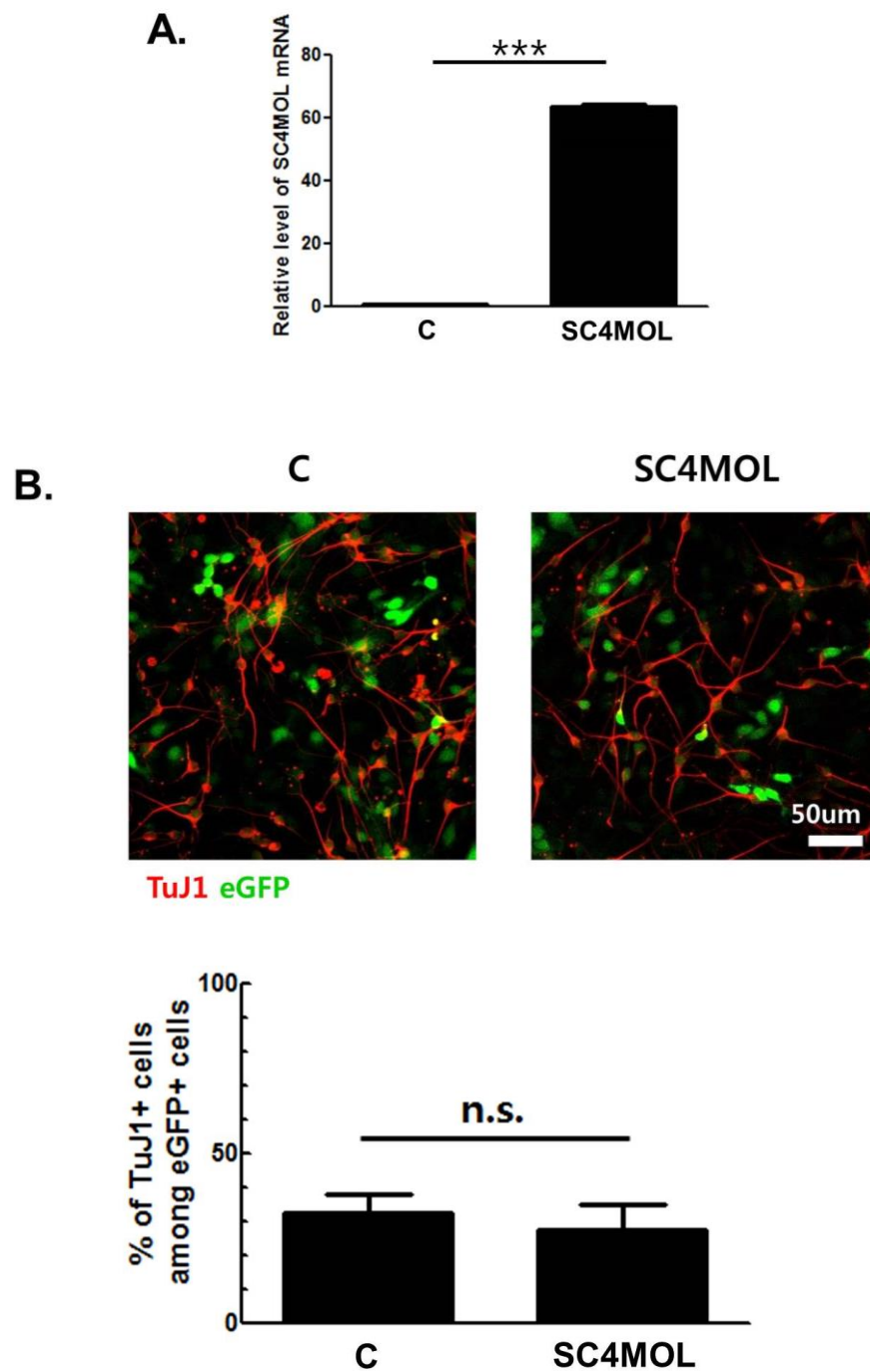
Primary NPCs were transduced with a retroviral vector expressing SC4MOL. The expression level of SC4MOL was increased by more than 63-fold compared with the control cells (Fig. III-8A). When SC4MOL-expressing NPCs were differentiated for 3 days, there was no statistically significant difference in the ratio of TuJ1-positive neurons between the two groups (Fig.III-8B), suggesting that



**Figure III-6. Effects of GSK3 isoforms on the neuronal differentiation of NPCs.** (A) Retroviral vectors expressing GSK3 $\alpha$  or GSK3 $\beta$  together with eGFP were added to NIH3T3 cells for 2 days. Total cell lysates were subjected to western blotting using antibodies to total GSK3 isoforms and tyrosine phosphorylated GSK3 isoforms. (B) NPCs were transduced with the same titer of respective retroviral vectors and then induced to differentiate for 3 days, followed by immunostaining for eGFP with TuJ1. Representative images of the staining and the percentages of each cell type among the eGFP-positive cells are shown. Scale bar, 50  $\mu$ m. \*\*P < 0.01; n.s., not significant; C, control.



**Figure III-7. Effects of IFN- $\gamma$  on the neuronal differentiation of NPCs.** (A,B) Retroviral vectors expressing IFN- $\gamma$  together with eGFP were added to NPCs for 2 days. Supernatants and cell lysates were prepared and subjected to ELISA and western blotting using antibodies to IFN- $\gamma$ , P-STAT1 (Tyr701) and  $\beta$ -actin. (C) NPCs were transduced with the same titer of respective retroviral vectors and then induced to differentiate for 3 days, followed by immunostaining for eGFP with TuJ1. Representative images of the staining and the percentages of each cell type among the eGFP-positive cells are shown. Scale bar, 50  $\mu$ m. \*\*\* $P < 0.001$ ; C, control.



**Figure III-8. Effects of SC4MOL on the neuronal differentiation of NPCs.** (A) Retroviral vectors expressing SC4MOL together with eGFP were added to NPCs for 2 days. qRT-PCR was performed using SC4MOL-specific primers. (B) NPCs were transduced with the same titer of respective retroviral vectors and then induced to differentiate for 3 days, followed by immunostaining for eGFP with TuJ1. Representative images of the staining and the percentages of each cell type among the eGFP-positive cells are shown. Scale bar, 50  $\mu$ m. \*\*\* $P < 0.001$ ; n.s., not significant; C, control

SC4MOL does not play an important role in embryonic neurogenesis.

### 3. Discussion

In this chapter, a rapid screening system using retroviral vectors and NPCs was employed to efficiently screen important factors in the context of embryonic neurogenesis. The factors used in this study were previously reported as being upregulated in the prenatally stressed embryonic brain, but their effects on neurogenesis have not been studied in the context of NPC differentiation. In experiments involving the overexpression of Nrf2, GSK3 $\alpha$ , and SC4MOL, the number of differentiated neurons was comparable with that in the control group, while this number was dramatically reduced when GSK3 $\beta$  or IFN- $\gamma$  was overexpressed. The results of these experiments suggest that Nrf2, GSK3 $\alpha$ , and SC4MOL do not play crucial roles in embryonic neurogenesis, whereas GSK3 $\beta$  and IFN- $\gamma$  strongly inhibit it. Gain-of-function system employed in this study have limitations as the endogenous expression of each gene might already be maximal, producing no additional effects when the level of expression is increased further. However, these data provided useful insights that can be used to conduct further investigations.

The process of neuronal differentiation is complex and only a small number of genes have been clearly demonstrated to be involved in the regulation of neurogenesis. The links between various signaling pathways have not been well understood and the underlying events during development that can result in serious neurological disorders are elusive. Thus, improved understanding of brain science and studies using NPCs may provide necessary information to facilitate the development of suitable therapies for patients with congenital/acquired brain disorders. If the factors involved in the differentiation of NPCs can be clearly identified and the nature of NPC regulation is further characterized, it will lead to a deeper understanding of brain pathology and possibly open up new approaches for the prevention or treatment of many brain disorders. According to the results described in this chapter, GSK3 $\beta$  and IFN- $\gamma$  are two factors that appeared to have negative effects on the



neuronal differentiation of NPCs. Thus, they were further characterized using various molecular techniques as described in the next two chapters.

## **CHAPTER IV**

### **Roles of GSK3 Isoforms in Embryonic Neurogenesis**

# 1. Background

GSK3 is a serine/threonine kinase that plays pivotal roles in many physiological processes including glucose metabolism, cell survival, proliferation, and differentiation [72]. In mammals, there are two isoforms of GSK3, GSK3 $\alpha$  and GSK3 $\beta$ , that share 98% homology in their kinase domain [73]. Although it is clear that GSK3 is involved in the proliferation and differentiation of NPCs [74], the exact roles of each isoform have not been clearly defined. No major brain malformations are found in GSK3 $\alpha$  KO mice or in mice engineered to have the GSK3 $\beta$  gene deleted in their NPCs [75], while the deletion of both the GSK3 $\alpha$  and GSK3 $\beta$  genes induces hyperproliferation and suppresses neuronal differentiation [76]. By contrast, mutant *Drosophila* that are defective in the *shaggy* gene, a GSK3 homologue, show increased neuronal differentiation [77]. The use of GSK3 inhibitors also promotes neuronal differentiation of human NPCs, rat ventral midbrain precursors, and rat neural stem cells [78-80]. Due to the conflicting results of these studies, the functions of GSK3 in the differentiation of NPCs and the exact effects of GSK3 $\alpha$  and GSK3 $\beta$  have been elusive.

GSK3 exists in cells in a constitutively active form, and its kinase activity is regulated by phosphorylation. The activity of GSK3 is downregulated when serine 21 of GSK3 $\alpha$  and serine 9 of GSK3 $\beta$  are phosphorylated, and upregulated when tyrosine residues (tyrosines 279 and 216 of GSK3 $\alpha$  and GSK3 $\beta$ , respectively) are phosphorylated [81]. Many kinases and phosphatases, such as protein kinase B (PKB), MAPK-activated protein kinase 1 (MAPKAP-K1), protein phosphatase (PP) 1, and PP2A, are known to influence the activity of GSK3 [82-84]. The mammalian target of rapamycin complex1 (mTORC1) is a kinase that has been reported to be involved in the serine phosphorylation of GSK3. In LPS-stimulated monocytes and tuberous sclerosis complex (TSC) 1 or TSC2 mutant embryonic fibroblasts, rapamycin has been found to block the serine phosphorylation of GSK3 $\beta$  [85, 86]. Although the relationship between GSK3 and mTORC1 is well defined in various situations such as cancer and inflammation, this relationship has not been delineated in the context of NPC differentiation. The activation of mTORC1, induced by insulin treatment or deletion of the TSC1 gene in embryonic telencephalic NPCs, results in premature differentiation, and rapamycin (an mTORC1

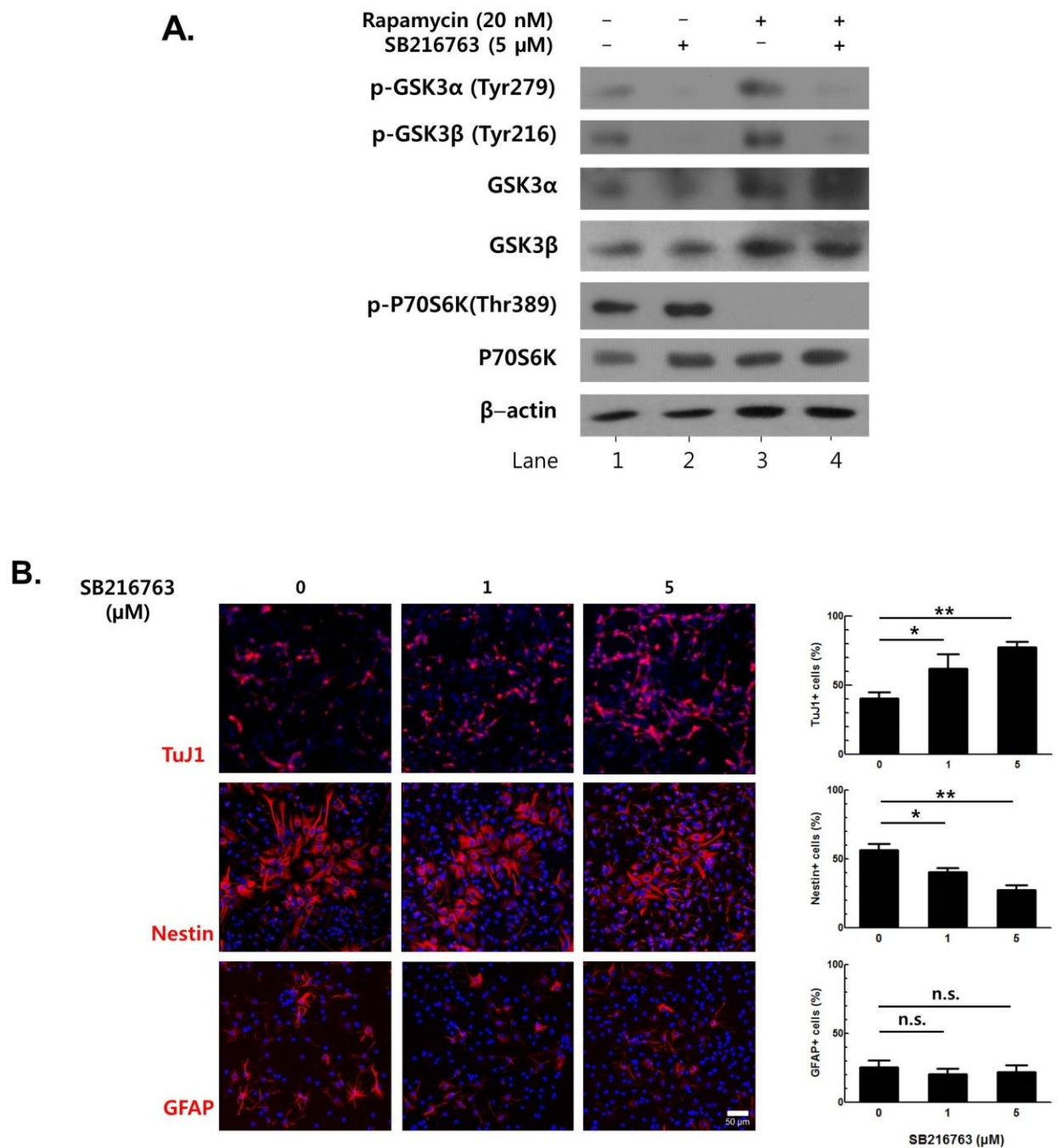
inhibitor) treatment inhibits these effects; these results suggest that mTORC1 has a role in neuronal differentiation [87, 88]. However, the downstream mechanism of mTORC1 in neurogenesis remains to be determined.

In this chapter, data obtained from *in vitro* and *in vivo* experiments involving E14.5 NPCs and E9 embryonic brains are described. These results indicate that GSK3 $\beta$  inhibits NPC differentiation. In addition, downregulation of GSK3 $\beta$  was found to de-repress the rapamycin-mediated inhibition of neuronal differentiation. Overall, these findings suggest that GSK3 $\beta$ , but not GSK3 $\alpha$ , is the isoform that negatively affects the differentiation of NPCs as the downstream target of the mTORC1 signaling pathway.

## **2. Results**

### **2.1 Effects of GSK3 inhibition on the neuronal differentiation of NPCs**

In several previous studies, chemical inhibitors of GSK3 have been used in attempts to define the roles of GSK3 [78-80]. To confirm the effect of GSK3 inhibition on the differentiation of NPCs, SB216763, an ATP-competitive inhibitor of GSK3 was used [89]. Primary NPCs were prepared from E14.5 mouse embryos and differentiated with DMEM containing 2% FBS and treated with 5  $\mu$ M SB216763 for 3 days. The effects of SB216763 on the tyrosine phosphorylation (an indicator of active GSK3) of each isoform [74, 90, 91] were observed by western blotting (Fig. IV-1A, compare lanes 1 and 2). The level of tyrosine phosphorylation of both GSK3 $\alpha$  and GSK3 $\beta$  were decreased 3 hr after 5  $\mu$ M SB216763 treatment, indicating the decreased activity of GSK3. Differentiated cells were then stained with specific markers including antibodies to TuJ1, Nestin (NPCs), and GFAP (astrocytes). Upon treatment with 5  $\mu$ M SB216763, the percentage of TuJ1-positive cells increased from 40% to 77%, but the percentage of Nestin-positive cells decreased from 57% to 28% (Fig. IV-1B). GFAP-positive cells were also counted to determine the effect of GSK3 on the formation of astrocytes, and no significant effect was observed. These data are consistent with

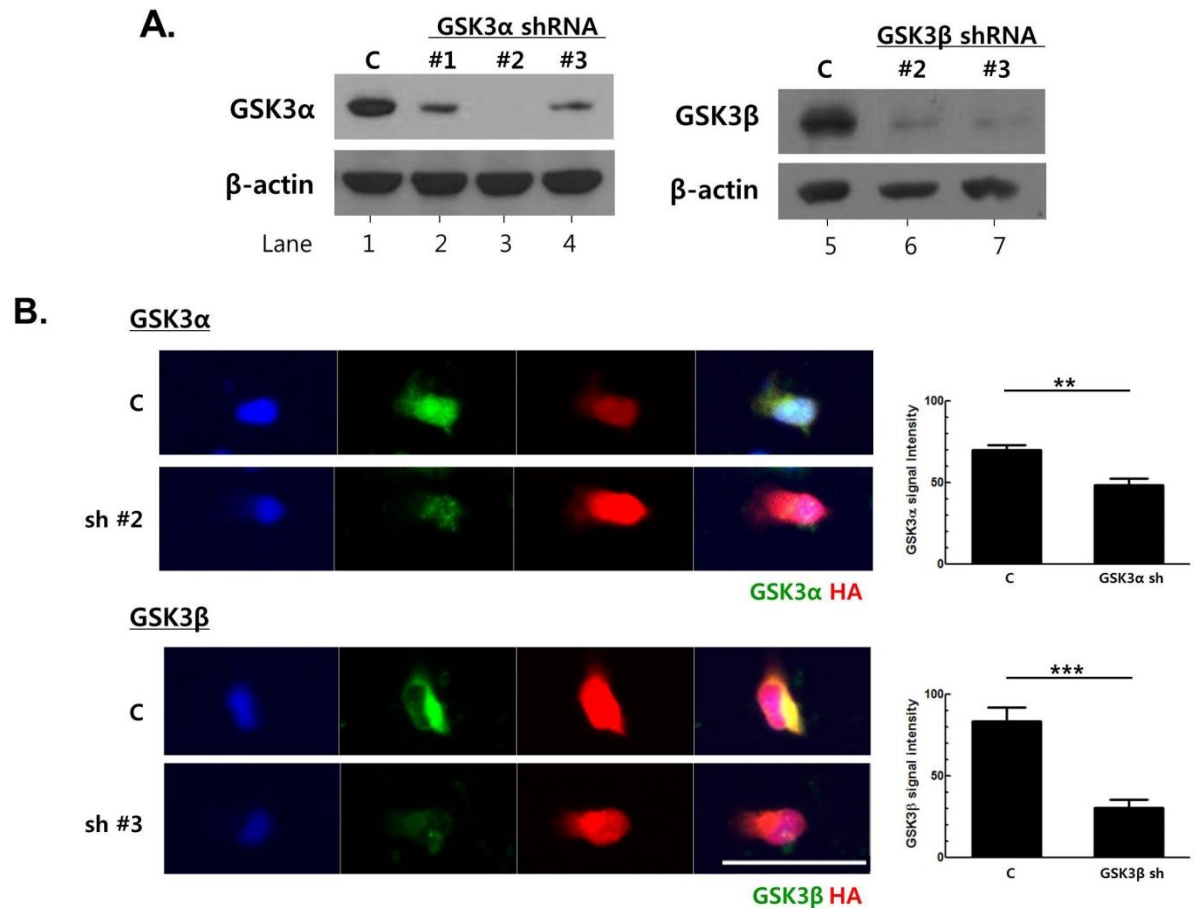


**Figure IV-1. Inhibition of GSK3 isoforms increases the neuronal differentiation of primary NPCs.** (A) Primary NPCs were induced to differentiate in the presence of 5 mM SB216763 and 20 nM rapamycin, separately or together, for 3 hr. Total cellular proteins were prepared and subjected to western blot. All proteins were analyzed using the same blot. (B) Primary NPCs were induced to differentiate with Dulbecco's modified Eagle's medium containing 2% fetal bovine serum in the presence of SB216763 for 3 days, followed by immunostaining for TuJ1, Nestin, and GFAP. Representative images of the staining and the percentages of each cell type are shown.

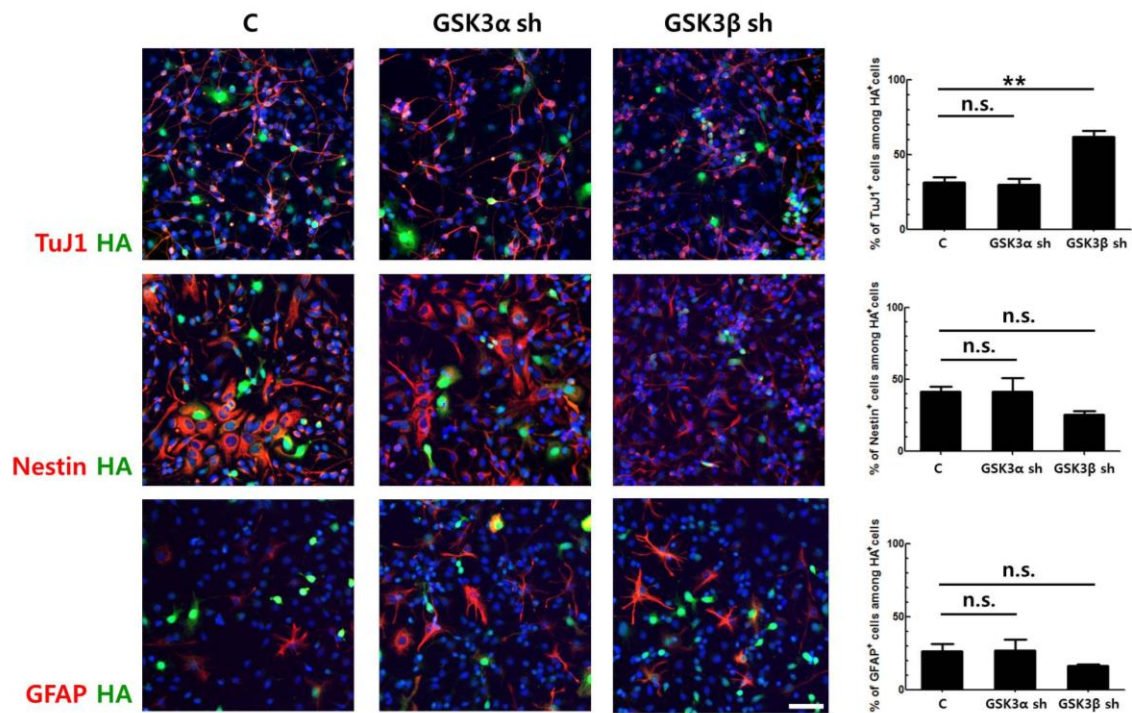
previous results in that the inhibition of both GSK3 $\alpha$  and  $\beta$  isoforms via chemical inhibitors enhanced the neuronal differentiation of progenitor cells, suggesting that GSK3 has a negative effect on neurogenesis.

To investigate which isoform of GSK3 contributes to the inhibition of neuronal differentiation, retroviral vectors expressing three different shRNA sequences against GSK3 $\alpha$  or GSK3 $\beta$ , together with HA tagged dsRED, were constructed. To determine knock-down efficiency, GSK3 $\alpha$ -overexpressing NIH3T3 cells and NIH3T3 cells were transduced with retroviral vectors expressing GSK3 $\alpha$  or GSK3 $\beta$  shRNA, respectively. GSK3 $\alpha$ -overexpressing NIH3T3 cells were used because the basal expression level of GSK3 $\alpha$  in NIH3T3 cells was undetectable. When the protein level of each isoform was measured after 2 days using Western blot, it was found that GSK3 $\alpha$  shRNA sequence #2 (Fig. IV-2A, lane 3) and GSK3 $\beta$  shRNA sequence #3 (Fig. IV-2A, lane 7) produced the highest knock-down efficiencies ( $98.9 \pm 1.5\%$  and  $71.3 \pm 10.9\%$  reduction compared to the control, respectively). To be certain, knock-down efficiency was also tested using primary NPCs. Cells were transduced with a retroviral vector expressing respective shRNAs, and 2 days later, the protein level was analyzed by immunostaining (Fig. IV-2B). When signal intensity was measured, the protein level of GSK3 $\alpha$  and GSK3 $\beta$  was decreased by  $30.6 \pm 12.7\%$  and  $63.5 \pm 15.2\%$ , respectively.

To investigate the effect of downregulation of GSK3 $\alpha$  and GSK3 $\beta$  on cell differentiation, primary NPCs were transduced with the same titer of the retroviral vectors expressing respective shRNAs and then induced to differentiate for 2 days. As DsRed fluorescence was too weak to be identified using a fluorescent microscope, the HA tag was stained, and the percentages of different cell markers among HA-positive cells were calculated (Fig. IV-3). In cells transduced with a control vector, approximately 31% of the transduced cells were TuJ1-positive. Interestingly, the number of TuJ1-positive cells among cells transduced with GSK3 $\alpha$  shRNA-expressing vector was similar to that of controls, while this number increased by approximately 2-fold in the GSK3 $\beta$  shRNA-expressing cells. Similarly, the proportion of Nestin-positive NPCs was decreased by nearly half when the expression of GSK3 $\beta$  was knocked-down. The changes in the percentages of GFAP-positive cells



**Figure IV-2. Knock-down efficiency of GSK3 shRNAs.** (A) GSK3 $\alpha$ -overexpressing NIH3T3 cells and NIH3T3 cells were transduced with retroviral vectors expressing GSK3 $\alpha$  shRNA and GSK3 $\beta$  shRNA, respectively. The knock-down efficiency of each shRNA sequence was analyzed by western blotting, using antibodies to GSK3 $\alpha$  and GSK3 $\beta$ . (B) Primary NPCs were transduced with retroviral vectors expressing GSK3 $\alpha$  shRNA No. 2 and GSK3 $\beta$  shRNA No. 3 for 2 days. The knock-down efficiency of each shRNA sequence was confirmed by immunostaining, using antibodies to HA tag with GSK3 $\alpha$  or GSK3 $\beta$ . The signal intensity of GSK3 $\alpha$  or GSK3 $\beta$  was quantified by ZEN 2012 (blue edition) software.



**Figure IV-3. Downregulation of GSK3 $\beta$ , but not GSK3 $\alpha$ , increases the neuronal differentiation of primary NPCs.** Primary NPCs were transduced with the same titer of respective retroviral vectors and then induced to differentiate for 2 days, followed by immunostaining for HA tag with TuJ1, Nestin, or GFAP. Representative images of the staining and the percentages of each cell type among HA-positive cells are shown. DNA was labeled with Hoechst 33258. Scale bar, 50  $\mu$ m. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001; n.s., not significant; C, control.



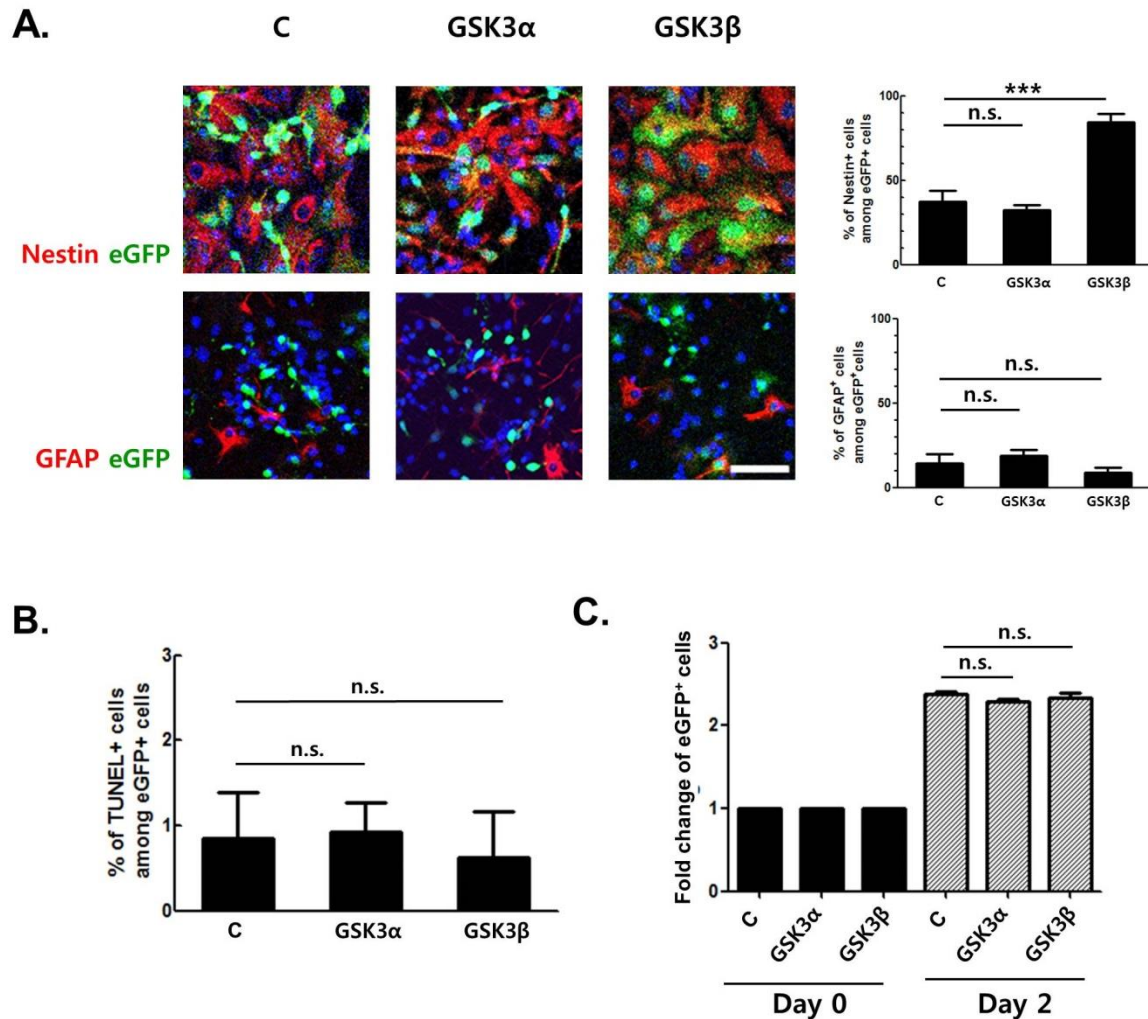
were not statistically significant. These data suggest that GSK3 $\alpha$  and GSK3 $\beta$  might have different effects on neuronal differentiation.

## **2.2 Effects of GSK3 overexpression on the differentiation and proliferation of NPCs**

In the previous chapter, it was demonstrated that overexpression of GSK3 $\beta$  inhibits the neuronal differentiation of NPCs. To further characterize the role of GSK3 isoforms in NPC differentiation, the percentage of other cell types including NPC and astrocyte were analyzed. Similar to the control, approximately 40% of the GSK3 $\alpha$ -transduced cells were positive for Nestin. However, the constitutive expression of GSK3 $\beta$  increased the percentage of Nestin-positive cells to 80%. The numbers of GFAP-positive cells were similar in all three groups (Fig.IV-4A).

To test whether the decrease in the percentage of TuJ1-positive cells resulted from NPC death, apoptotic cell death was analyzed. NPCs were transduced with GSK3 $\alpha$ - and GSK3 $\beta$ -overexpressing vectors for 2 days and differentiated for 3 days. The cells were then subjected for a TUNEL assay. As shown in Fig. IV-4B, the numbers of TUNEL-positive cells were comparable between the control, GSK3 $\alpha$ -, and GSK3 $\beta$ -transduced cells, indicating that apoptosis did not have a significant influence.

The effect of GSK3 isoforms on the proliferation of NPCs was also examined by FACS analysis. Primary NPCs were transduced with respective retroviral vectors, and 2 days later, changes in the number of GFP<sup>+</sup> NPCs were measured (Fig.IV-4C). In all three groups, the number of GFP<sup>+</sup> cells was doubled to a similar level by day 2, and there was no significant difference between the control and GSK3 $\alpha$ - and GSK3 $\beta$ - overexpressing cells. These data indicate that GSK3 does not influence the proliferative potential of NPCs. Taken together, abovementioned results corroborate the notion that GSK3 $\beta$ , but not GSK3 $\alpha$ , inhibited neuronal differentiation and helped NPCs to maintain their stemness.

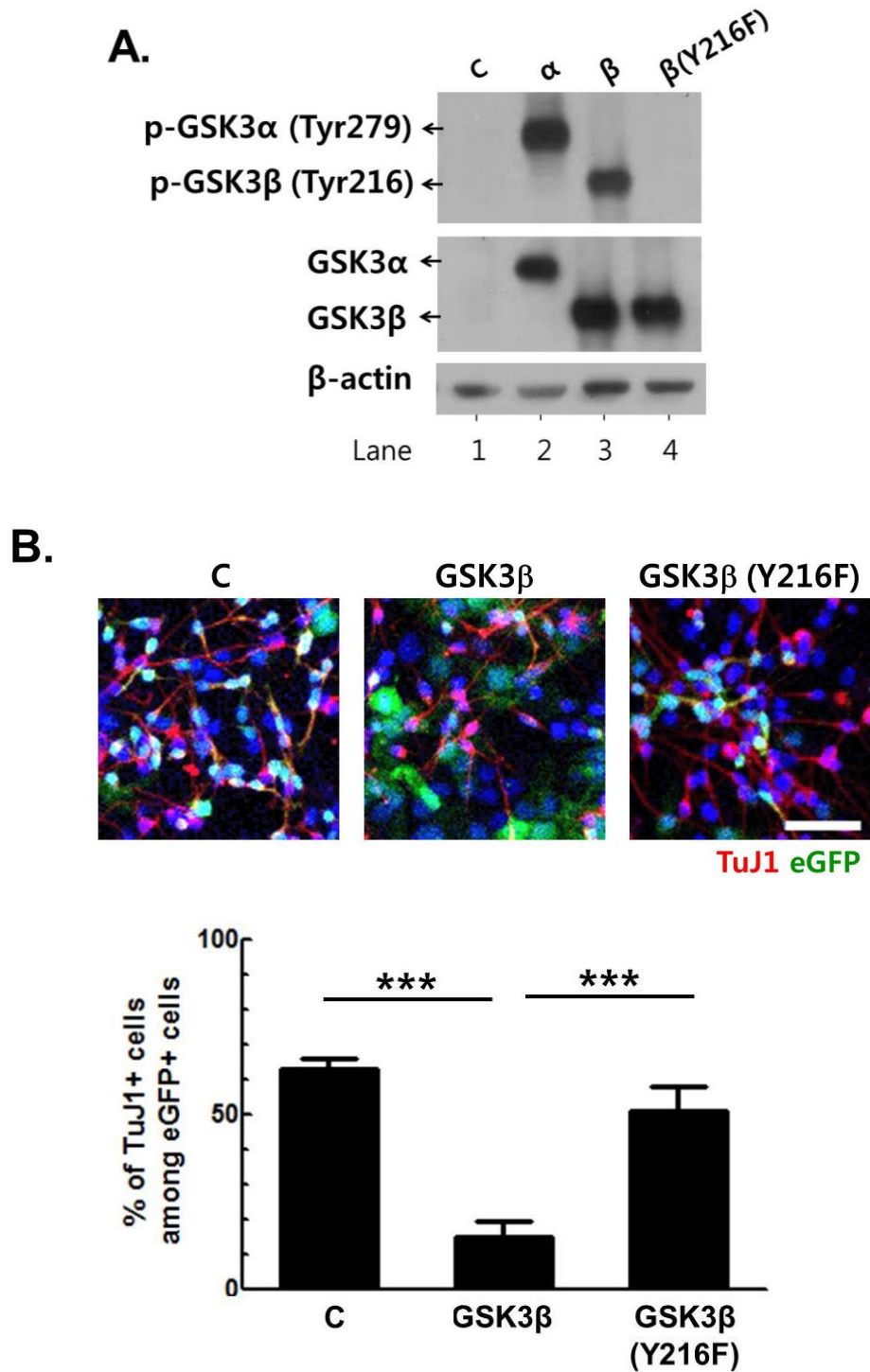


**Figure IV-4. GSK3 $\beta$ , but not GSK3 $\alpha$ , suppresses the neuronal differentiation of NPCs without affecting cellular apoptosis and NPC proliferation.** (A) Primary NPCs were transduced with the same titer of respective retroviral vectors and then induced to differentiate for 3 days. The cells were subjected to immunostaining for eGFP and Nestin, or GFAP. Representative images of the staining and the percentages of each cell type among the eGFP-positive cells are shown. (B) Two days after the transduction of primary NPCs with the above retroviral vectors, they were subjected to differentiate for 3 days, and apoptotic cell death was analyzed by a TUNEL assay. (C) Two days after the transduction of primary NPCs with above retroviral vectors, the % of GFP+ cells were analyzed by fluorescence-activated cell sorting for 2 days.

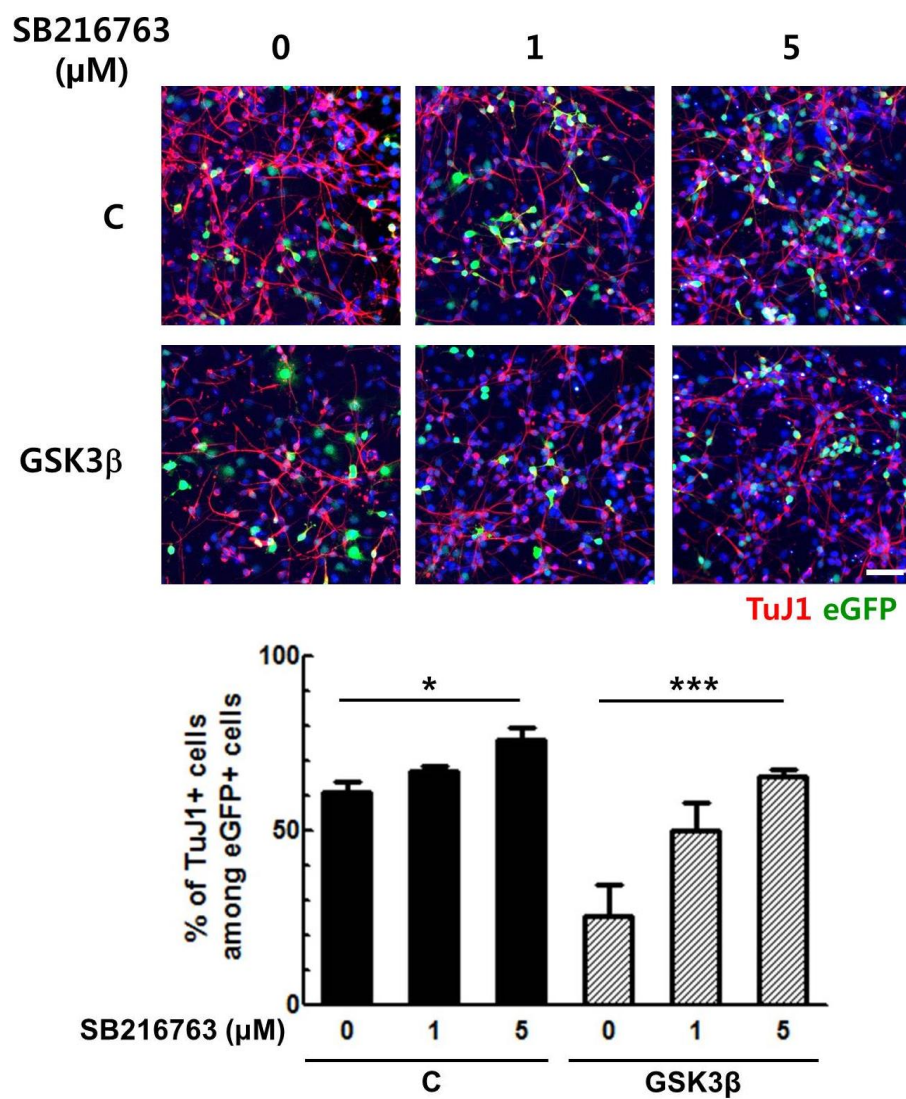
To determine whether the kinase activity of GSK3 $\beta$  is involved in the inhibition of neuronal differentiation, a retroviral vector expressing a mutant form of GSK3 $\beta$  that contains phenylalanine in the place of tyrosine at the 216<sup>th</sup> codon was constructed. This mutant form has previously been shown to exhibit reduced kinase activity [90], and its expression was confirmed by western blotting as shown in Fig. IV-5A (lane 4). Primary NPCs were transduced with retroviral vectors expressing the wild type or this mutant sequence of GSK3 $\beta$  and induced to differentiate for 3 days. Retroviral expression of this mutant form resulted in a significantly greater number of TuJ1-positive cells compared to cells expressing the wild-type protein (Fig. IV-5B). When GSK3 $\beta$ -expressing progenitor cells were induced to differentiate in the presence of SB216763 for 3 days, the GSK3 $\beta$ -mediated inhibition of neuronal differentiation was abrogated in a dose-dependent manner (Fig. IV-6). These data demonstrate that GSK3 $\beta$  negatively controls the neuronal differentiation of progenitor cells and that the kinase activity of GSK3 $\beta$  is essential to this inhibition.

## **2.3 Effects of GSK3 $\beta$ on neuronal differentiation in the embryonic brain**

To test the effect of GSK3 $\beta$  on neuronal differentiation during brain development *in vivo*, a retroviral vector expressing GSK3 $\beta$  and eGFP as a bicistronic message was delivered to the ventricles of E9.5 embryonic brains using the UIGD technique [53], and the brains were analyzed at E14.5. Under this experimental condition, NPCs in the VZ are transduced with retroviral vectors, and the neurons produced from these NPCs migrate away from the VZ during neurogenesis. Because the NICD is well known to strongly inhibit neuronal differentiation [52, 92], this sequence was used as a positive control. When a control vector expressing only eGFP was injected into embryonic brains, approximately 86% of eGFP-positive cells were found in the TuJ1-stained region, while NICD-transduced cells were predominantly localized in the VZ (Fig. IV-7A and B). In the case of GSK3 $\beta$ , approximately 31% of cells were positive for TuJ1, and this percentage was significantly lower than that of the control. These data suggest that GSK3 $\beta$  can indeed suppress neuronal differentiation and



**Figure IV-5. The kinase activity of GSK3β is required for the suppression of neurogenesis. (A)** Retroviral vectors expressing GSK3α, GSK3β, or GSK3β (Y216F), together with eGFP, were used to transduce NIH3T3 cells. The level of expression and tyrosine phosphorylation of GSK3α, GSK3β, or GSK3β(Y216F) was analyzed by western blotting, using specific antibodies. **(B)** Retroviral vectors expressing wild-type GSK3β or mutant GSK3β (Y216F) were used to transduce primary NPCs, followed by differentiation for 3 days. Representative images of eGFP and TuJ1 staining are shown.



**Figure IV-6. GSK3 $\beta$  negatively controls the neuronal differentiation of NPCs.** Primary NPCs were transduced with a retroviral vector expressing GSK3 $\beta$ , and then induced to differentiate for 3 days in the presence of SB216763. The cells were double labeled with antibodies to eGFP and TuJ1. The percentages of TuJ1-positive cells among the eGFP-positive cells are presented. DNA was labeled with Hoechst 33258. Scale bar, 50  $\mu$ m. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; n.s., not significant; C, control.



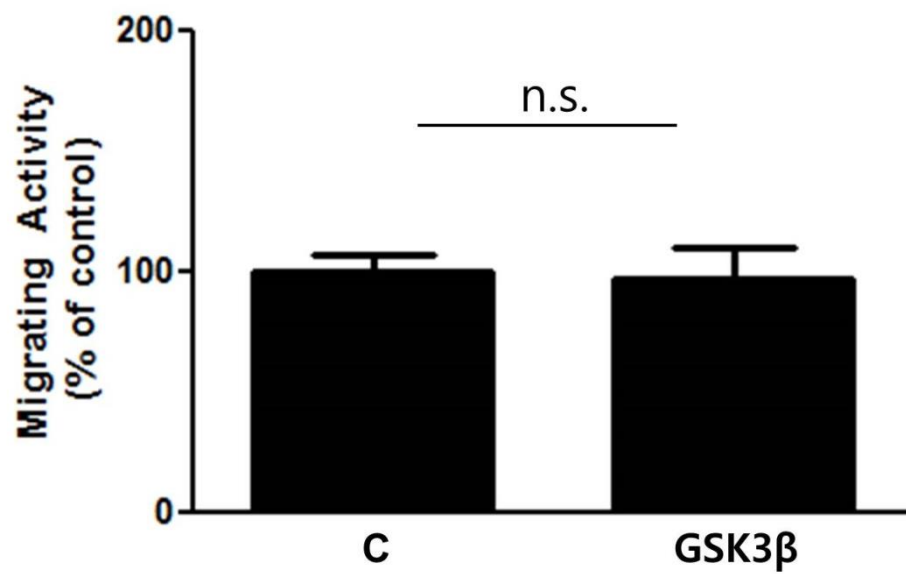


that it was involved in maintaining the characteristics of NPCs in embryonic brains.

To determine whether the retention of NPCs in the VZ in the GSK3 $\beta$  group resulted from the inhibition of cell migration, the effects of GSK3 $\beta$  overexpression on the migration of NPCs were also tested. Primary NPCs were transduced with the control or GSK3 $\beta$ -expressing retroviral vectors. After 2 days, eGFP-positive cells were sorted and migrating ability was measured by transwell migration assay (Fig. IV-8). There was no statistically significant difference between the control and GSK3 $\beta$ -overexpressing cells, suggesting that GSK3 $\beta$  does not affect the migrating ability of NPCs.

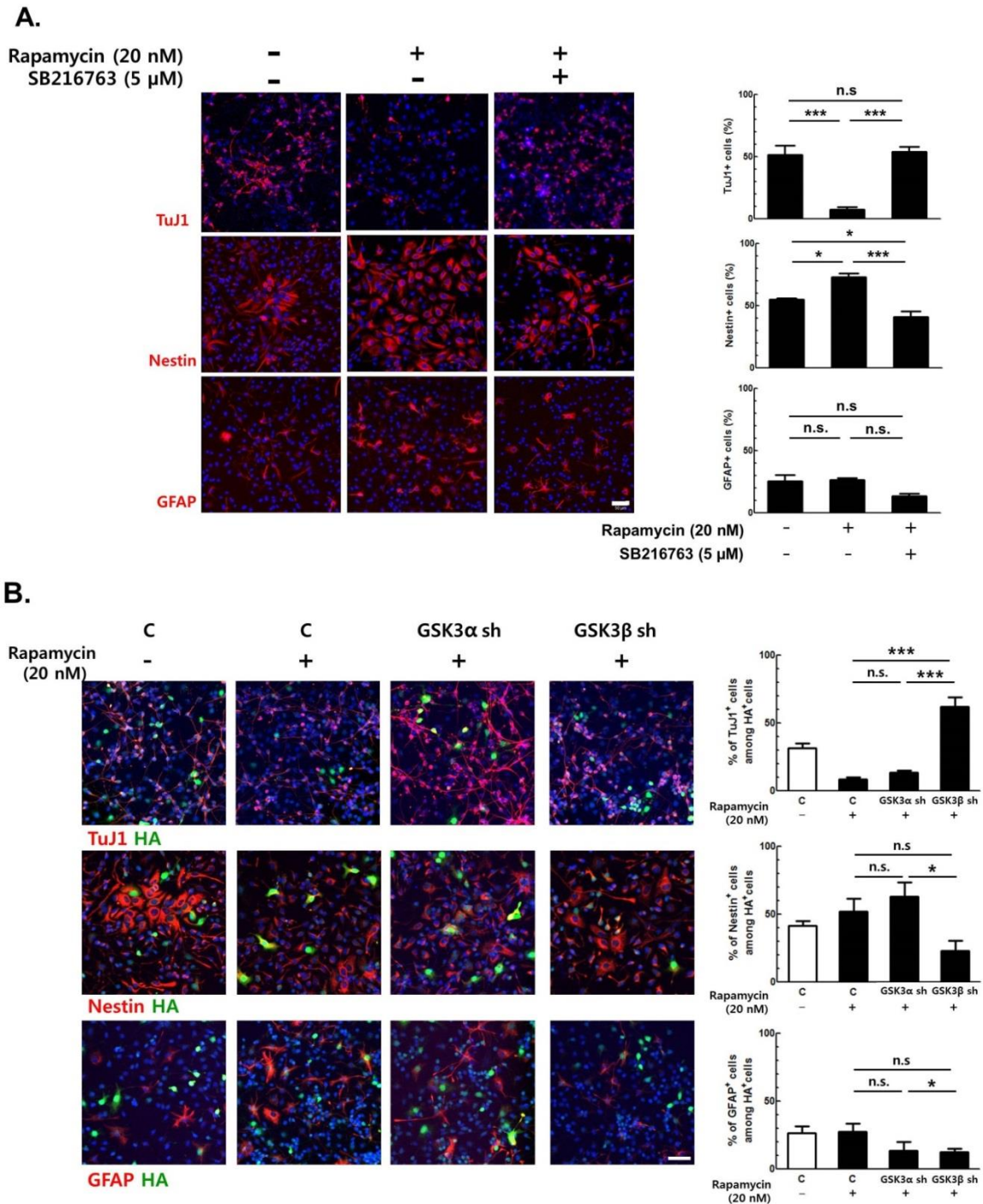
## **2.4 Regulation of GSK3 by the mTORC1 signaling pathway**

Rapamycin, an mTORC1 inhibitor, has been reported to repress the neuronal differentiation of NPCs [87, 88, 93, 94]. In the following experiments, it was tested whether mTORC1 is involved in the regulation of GSK3 in NPCs using chemical inhibitors. Primary E14.5 NPCs were differentiated with DMEM containing 2% FBS and treated with 20 nM rapamycin alone or with 5  $\mu$ M SB216763 for 3 days. The activities of SB216763 and rapamycin were confirmed by measuring the phosphorylation level of their downstream target proteins, p-GSK3 $\alpha$  (Tyr279), p-GSK3 $\beta$  (Tyr216) and p-P70S6K (Thr389), using Western blot analysis (Fig. IV-1A, lanes 2 and 3). Upon SB216763 and rapamycin treatment for 3 hr, tyrosine phosphorylation of both GSK3 isoforms and p-P70S6K levels were decreased, respectively. When cells were treated with both inhibitors, the level of the downstream targets of both inhibitors was decreased. These data showed that rapamycin did not influence the inhibitory effect of SB216763 or vice versa (Fig. IV-1A, lane 4). Upon 20 nM rapamycin treatment, the percentage of TuJ1-positive cells was reduced from 51% to 8%, but 5  $\mu$ M SB216763 completely reversed this effect (Fig. IV-9A). A similar observation was made in the C17.2 murine immortalized neural stem cell line using SB216763 and LiCl (another well-known GSK3 inhibitor) (data not shown). Again, the proportion of Nestin-positive cells increased from 55% to 73% after rapamycin treatment, and the extent of this increase was reduced by SB216763 co-treatment. The percentage of GFAP-positive cells was not affected by rapamycin or SB216763. These data suggest that GSK3 might inhibit neuronal differentiation as a downstream target of mTORC1.



**Figure IV-8. GSK3 $\beta$  does not affect the migrating ability of NPCs.** Control or GSK3 $\beta$ -overexpressing NPCs were sorted, and their migrating ability was measured using transwell migration assay. The numbers of cells that migrated to the bottom of the membrane were counted. Scale bar, 100  $\mu$ m. \*\*\* $P < 0.001$ ; C, control.

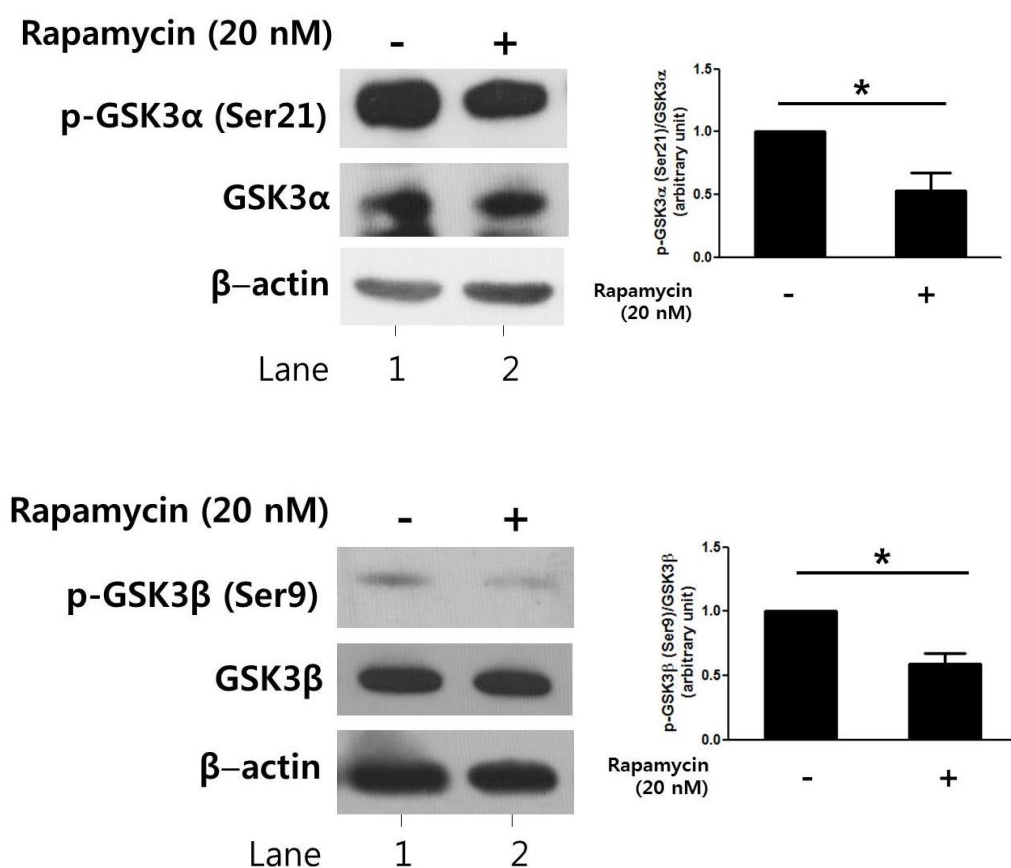




**Figure IV-9. mTORC1 inhibits GSK3 $\beta$ .** (A) Differentiated primary NPCs in the presence of 5 mM SB216763 and 20nM rapamycin, separately or together, were immunostained using antibodies to TuJ1, Nestin, and GFAP. Representative images of the staining and the results from the cell counting are shown. (B) Primary NPCs were transduced with retroviral vectors expressing control shRNA, GSK3 $\alpha$  shRNA or GSK3 $\beta$  shRNA for 2 days, followed by differentiation with or without rapamycin for 2 days. Cells were immunostained for HA tag with TuJ1, Nestin, or GFAP. Representative images of the staining and the percentages of each cell type among HA-positive cells are shown.

It was hypothesized that GSK3 $\alpha$  and GSK3 $\beta$  might be differentially regulated by mTORC1. Because SB216763 inhibits both GSK3 $\alpha$  and GSK3 $\beta$ , shRNAs targeting each isoform were employed to test the effect of mTORC1 on each isoform. Primary NPCs were transduced with retroviral vectors expressing shRNA against GSK3 $\alpha$  or GSK3 $\beta$  and induced to differentiate in the presence of 20 nM rapamycin for 2 days (Fig. IV-9B). Similar to the above results, rapamycin treatment reduced the number of immature neurons by approximately 65% in the control group (from 31.5% to 11.2%). The level of reduction was similar when GSK3 $\alpha$  shRNA-expressing cells were treated with rapamycin. However, this reduction was not observed in the GSK3 $\beta$  shRNA-expressing group. As expected, knock-down of the expression of GSK3 $\beta$  produced a proportion of TuJ1- positive cells that was greater than that of the control group (Fig. IV-9B, white bar), similar to the result shown in Fig. IV-3. The proportion of Nestin-positive cells was increased approximately 1.5-fold when control and GSK3 $\alpha$ -silenced cells were treated with rapamycin. When GSK3 $\beta$  expression was lowered by shRNA, this increase was significantly reduced, and only approximately 23% of the GSK3 $\beta$  shRNA-expressing cells remained as NPCs. Therefore, these data not only confirm that rapamycin inhibited neuronal differentiation by blocking the repression of GSK3 activity but also clearly demonstrate that GSK3 $\alpha$  and GSK3 $\beta$  were differentially affected by the upstream regulator mTORC1.

As mTORC1 is known to regulate GSK3 via phosphorylation of the inhibitory serine residue [85, 86], the effects of mTORC1 on serine phosphorylation of GSK3 $\alpha$  and GSK3 $\beta$  were investigated in NPCs. Primary NPCs were treated with 20 nM rapamycin for 1 day and proteins were prepared and analyzed by western blotting. The serine phosphorylation levels of both GSK3 $\alpha$  and GSK3 $\beta$  were decreased by 50.7% and 37.6% upon rapamycin treatment, respectively (Fig. IV-10). These data indicate that the inhibition of mTORC1 resulted in reduced serine phosphorylation of both GSK3 $\alpha$  and GSK3 $\beta$ , suggesting that other regulatory mechanisms might be involved in controlling the activity of GSK3 isoforms downstream of mTORC1.



**Figure IV-10. Serine phosphorylation of both GSK3α and GSK3β are reduced upon the inhibition of mTORC1.** Proteins from the primary NPCs induced to differentiate in the presence of rapamycin for 1 day were prepared and subjected to western blot using antibodies to p-GSK3α (Ser21) and p-GSK3β (Ser9). The band intensity was quantified by Image J software. DNA was labeled with Hoechst 33258. Scale bar, 50 μm. \*P < 0.05; \*\*\*P < 0.001; n.s., not significant; C, control.

### 3. Discussion

Prior to this study, the roles of the two isoforms of GSK3, GSK3 $\alpha$  and GSK3 $\beta$ , in the differentiation of NPCs were unclear. To identify the functions of each isoform in brain development, the expression levels of GSK3 $\alpha$  and GSK3 $\beta$  were manipulated separately in NPCs through the downregulation or overexpression of each isoform. Knock-down of GSK3 $\beta$  expression led to increased neurogenesis, while stable expression of GSK3 $\beta$  suppressed neurogenesis and helped to maintain NPC characteristics both *in vitro* and *in vivo*. By contrast, GSK3 $\alpha$  alone did not produce any noticeable effects. These data are in agreement with the results of experiments involving *Drosophila* that are defective in the *shaggy* gene, a GSK3 homologue, which exhibit bristle hyperplasia on the thorax [77].

These results contrast with the findings of a double KO study by Kim *et al.* [76] which showed that the loss of GSK3 induces hyperproliferation of NPCs and decreases neuronal differentiation. Although the reason for the discrepancy between these two studies is not clear, the germ-line deletion of GSK3 $\alpha$  might have caused indirect effects, as GSK3 is an important regulator during embryogenesis [95]. Both GSK3 $\alpha$  and GSK3 $\beta$  are known to be expressed from the two-cell stage embryo, and their activities are tightly controlled as the embryo develops [96]. Therefore, the germ-line manipulation of either form of GSK3 may have produced unexpected outcomes in the later stages of development, especially when the other form, which could have compensated for the loss, was deleted. In this study, I controlled the expression level of each isoform at a relatively late stage of embryonic development and demonstrated that GSK3 $\beta$  acts as a negative regulator during embryonic neurogenesis.

The results described in this chapter suggest that the two isoforms can have two different consequences in the context of neuronal differentiation. Considering the different life spans of GSK3 $\alpha$  and GSK3 $\beta$  single KO mice [76, 97, 98], it is possible that GSK3 $\alpha$  may not be able to complement all the defects caused by the absence of GSK3 $\beta$ . This possibility could indicate that the two isoforms target different populations of cellular proteins. GSK3 has been shown to play roles in many different

signaling pathways that are involved in neural cell proliferation and differentiation including Wnt, Shh, Notch, and c-Myc [99-103]; however, all of the studies that demonstrated these effects were performed only with GSK3 $\beta$ . Thus, GSK3 $\alpha$  might not target the same proteins as GSK3 $\beta$ , and even if it does, the regulatory activities of each isoform may be manipulated differently.

The exact mechanisms by which GSK3 $\alpha$  and GSK3 $\beta$  are regulated require further investigation. In this study, rapamycin failed to suppress neuronal differentiation in GSK3 $\beta$ -, but not GSK3 $\alpha$ -, knock-down cells, indicating that mTORC1 might not affect the activity of GSK3 $\alpha$ . Other investigators have indicated that the functions of GSK3 $\alpha$  and GSK3 $\beta$  can be regulated independently. For example, dephosphorylation of the N-terminal regions of the two isoforms has been shown to be regulated by different phosphatases, PP2A for GSK3 $\alpha$  and PP1 for GSK3 $\beta$ . It was also reported that GSK3 $\alpha$  was preferentially localized in the nucleus compared to GSK3 $\beta$ , which might allow GSK3 $\alpha$  to be more competent in phosphorylating its target protein [104]. Whatever the case, these data unequivocally demonstrate that GSK3 $\beta$ , but not GSK3 $\alpha$ , is a key mediator of NPC differentiation.

A recent publication showed that the selective silencing of either GSK3 $\alpha$  or GSK3 $\beta$  leads to different consequences in mouse models of Alzheimer's disease [105]. GSK3 $\alpha$ -, but not GSK3 $\beta$ -, silenced amyloid precursor protein (APP) transgenic mice exhibited reduced levels of amyloid  $\beta$  formation in the adult hippocampus, which indicates the characteristic roles of GSK3 $\alpha$  and GSK3 $\beta$  in neurodegenerative disease. GSK3 is known to be a critical protein involved in numerous central nervous system diseases including bipolar disorder, Alzheimer's disease, and schizophrenia [106-108]. Thus, the study of GSK3 isoforms is critical not only for the understanding of these serious human disorders but also for the development of new and effective therapeutic strategies.

## **CHAPTER V**

# **Effects of IFN- $\gamma$ on Embryonic Neurogenesis**

# 1. Background

IFN- $\gamma$  is one of the inflammatory cytokines expressed in the fetal brain following an inflammatory stimulus [109]. It can be expressed from E7 in mice and week 21 of pregnancy in humans [110, 111]. The binding of IFN- $\gamma$  to its receptor results in the phosphorylation of JAK1 and JAK2, and the subsequent phosphorylation of STAT1. Phosphorylated STAT1 then forms a homodimer and translocates to the nucleus [112]. STAT1, acting as a transcription factor, can positively or negatively regulate downstream target genes, depending on the presence of other cofactors [113].

The effects of IFN- $\gamma$  on neurogenesis have been investigated *in vitro* using recombinant IFN- $\gamma$  in various types of cells. However, depending on the cell type and the concentration of IFN- $\gamma$ , different results have been reported. In experiments involving murine adult neural stem cells, a human neuroblastoma cell line, a neonatal NPC line (C17.2), and E15-E16 neurons, treatment with IFN- $\gamma$  resulted in increased neurogenesis [114-117]. However, in an experiment involving cells from the subventricular zone of postnatal day 2 mouse brains, IFN- $\gamma$  had the opposite effect [118]. Furthermore, it was also reported that a high level of IFN- $\gamma$  resulted in irregular cell types double-positive for GFAP and TuJ1 when E14 neural stem/precursor cells were used [119]. Not only are the effects of IFN- $\gamma$  on neurogenesis controversial, but the downstream mechanism of this cytokine is barely elucidated.

In research described in this chapter, the role of IFN- $\gamma$  in embryonic neurogenesis was investigated both *in vitro* and *in vivo*, using primary E14.5 NPCs and E9.5 embryonic brains, respectively. It was found that IFN- $\gamma$  effectively inhibits neurogenesis, and this inhibition was dependent on the JAK/STAT1 signaling pathway in primary NPCs. An *in vivo* study using E14.5 embryonic brains also showed a similar result. Interestingly, IFN- $\gamma$  treatment specifically decreased the RNA level of *Neurogenin 2* (*Neurog2*) among other proneural genes.

# 2. Results

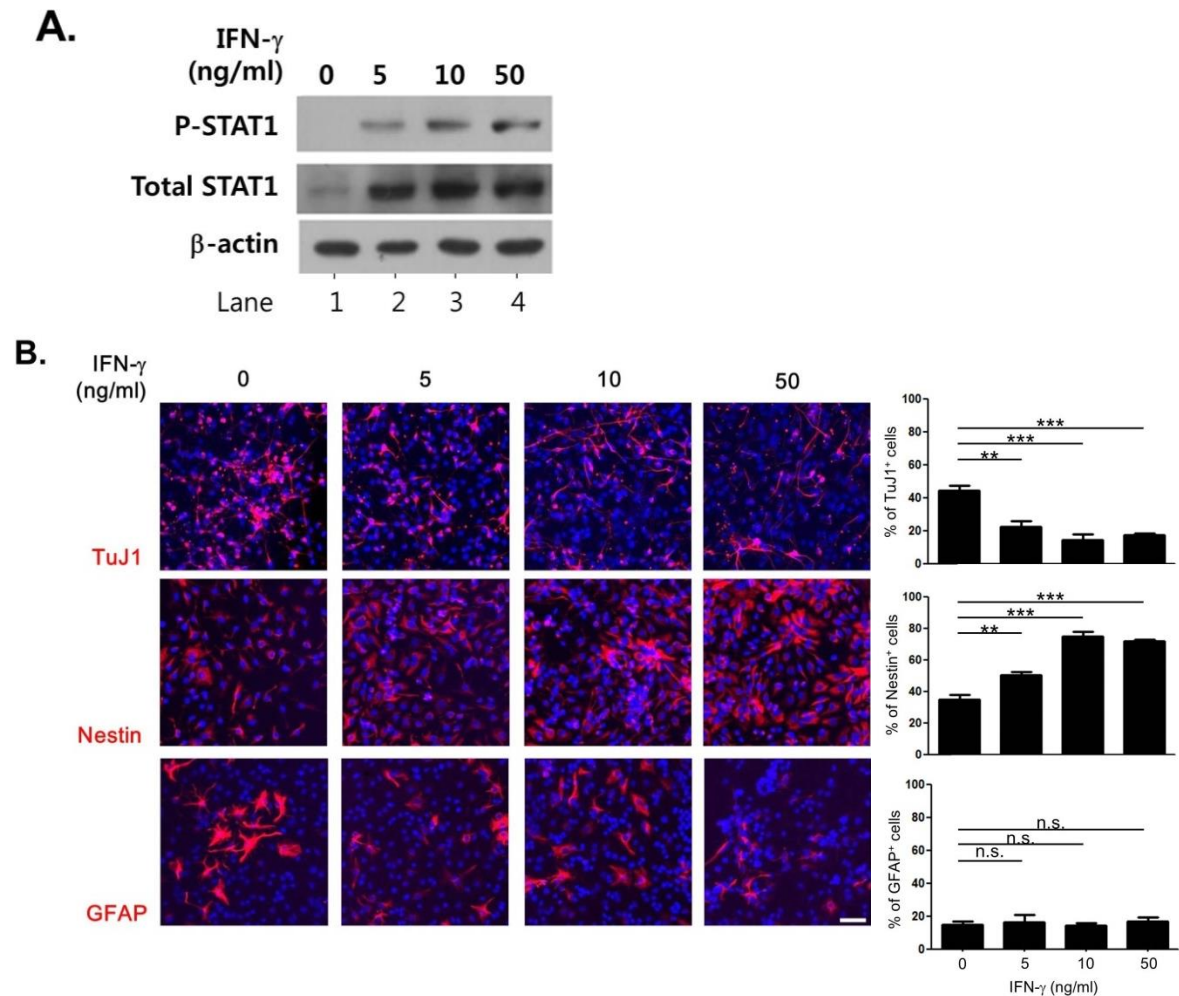
## 2.1. Effects of IFN- $\gamma$ on the neuronal differentiation of NPCs

Overexpression of IFN- $\gamma$  in NPCs via retroviral transduction process could be problematic as the transduction efficiency and amount of IFN- $\gamma$  released by each experimental group may vary every time. Thus, to investigate the effects of IFN- $\gamma$  on neuronal differentiation in a more controllable manner, recombinant IFN- $\gamma$  was used. Primary NPCs were prepared from E14.5 mouse embryos and cultured in NPC proliferation medium. After 2 days, NPCs were differentiated with DMEM containing 2% FBS in the presence of 5-50 ng/mL IFN- $\gamma$  for another 2 days. To test the functionality of IFN- $\gamma$  used in this study, the status and level of STAT1 were analyzed. As expected, the amount of Tyr701-phosphorylated STAT1 and the amount of total STAT1 increased as the concentration of IFN- $\gamma$  increased, indicating that the recombinant IFN- $\gamma$  is active (Fig.V-1A). Differentiated cells were stained for specific markers of immature neurons (TuJ1), NPCs (Nestin), and astrocytes (GFAP). 45 $\pm$ 5.2% of total cells were TuJ1-positive immature neurons, while 35 $\pm$ 5.3% of them were Nestin-positive NPCs. Only a small proportion of cells (15 $\pm$ 1.2%) were GFAP-positive astrocytes. Upon treatment with 50 ng/mL IFN- $\gamma$ , the percentage of TuJ1-positive cells was decreased to 17 $\pm$ 2.0%, while the percentage of Nestin-positive cells was increased to 72 $\pm$ 1.2%. There was no significant difference in the percentage of GFAP-positive astrocytes (Fig. V-1B). Similar to the results from Chapter III, these results strongly suggest an inhibitory role of IFN- $\gamma$  in the neuronal differentiation of NPCs.

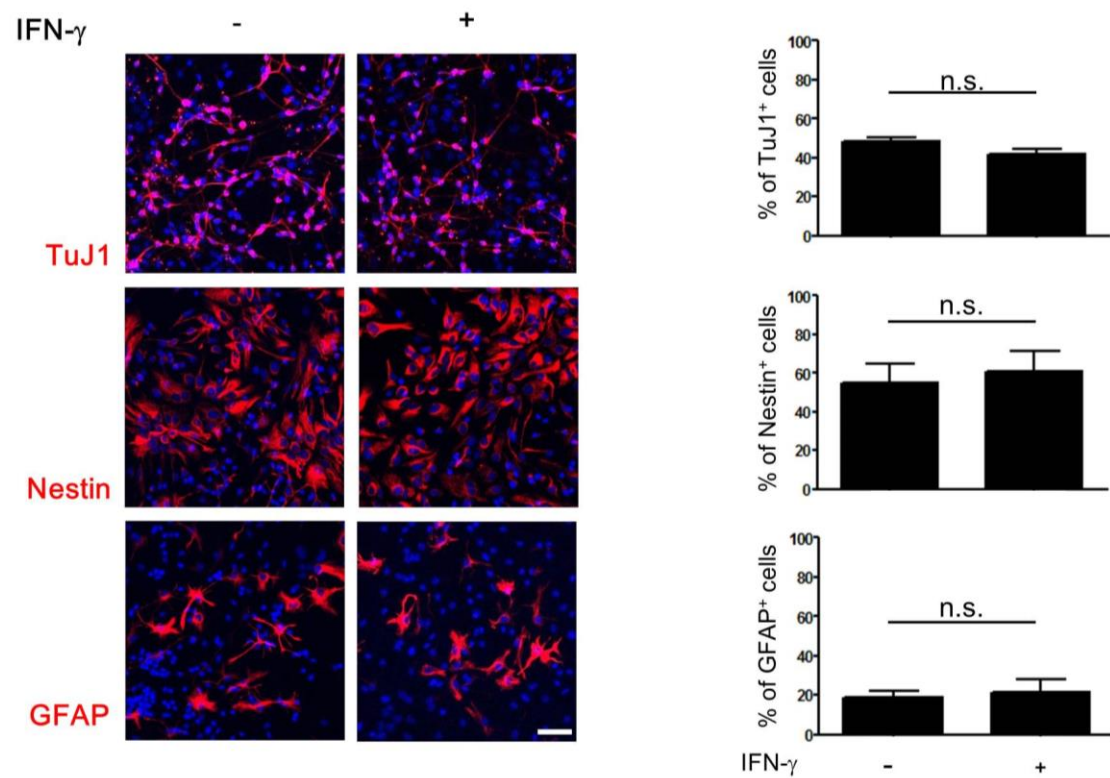
To test whether IFN- $\gamma$  has an effect on NPCs in a proliferative status, isolated NPCs were grown in NPC proliferation medium with 50 ng/mL IFN- $\gamma$  for 2 days and differentiated in the absence of IFN- $\gamma$ . There were no statistically significant differences in the percentages of different cell types between the control and IFN- $\gamma$ -treated cells (Fig. V-2). This indicates that IFN- $\gamma$  may have effects only on NPCs that have started differentiation and not on actively proliferating NPCs.

To investigate whether the decreased number of TuJ1-positive cells was due to increased cell





**Figure V-1. IFN- $\gamma$  decreases the neuronal differentiation of primary NPCs.** (A) NPCs were differentiated with various concentrations of IFN- $\gamma$ . After 3 hr, whole cell lysates were prepared and the level of phosphorylated STAT1 was observed by western blotting using antibodies to P-STAT1 (Tyr701), total STAT1 and  $\beta$ -actin. (B) Primary NPCs were induced to differentiate in the presence of different concentrations of IFN- $\gamma$  for 2 days and then immunostained for different cell markers (TuJ1, Nestin, and GFAP). Representative images of the staining and the percentages of each cell type are shown.



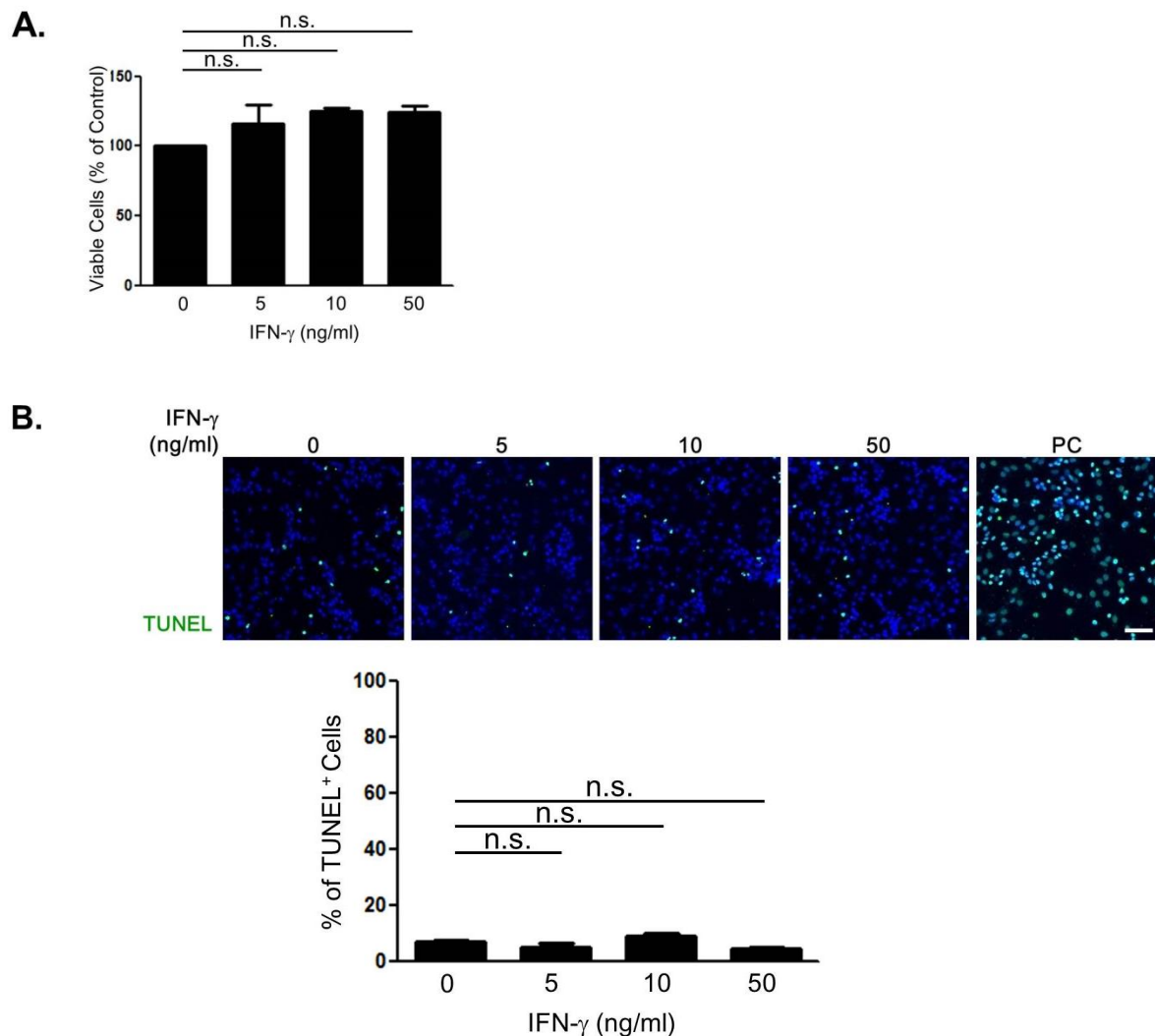
**Figure V-2. IFN- $\gamma$  has no effect on proliferating NPCs.** Primary NPCs were grown in proliferation medium in the presence of 50 ng/mL IFN- $\gamma$  for 2 days and induced to differentiate in the absence of IFN- $\gamma$ . After 2 days, immunostaining was done for TuJ1, Nestin, and GFAP. Representative images of the staining and the percentages of each cell type are shown.

death, NPCs were differentiated with 5-50 ng/mL IFN- $\gamma$  for 2 days and subjected to MTT and TUNEL assays. As shown in Fig.V-3A, no statistically significant difference was found in the number of viable cells between the control and IFN- $\gamma$ -treated groups. Moreover, similar numbers of the TUNEL-positive cells were observed in the control and IFN- $\gamma$ -treated groups (Fig. V-3B). These data demonstrate that IFN- $\gamma$  does not have cellular toxicity and does not have an effect on apoptotic cell death.

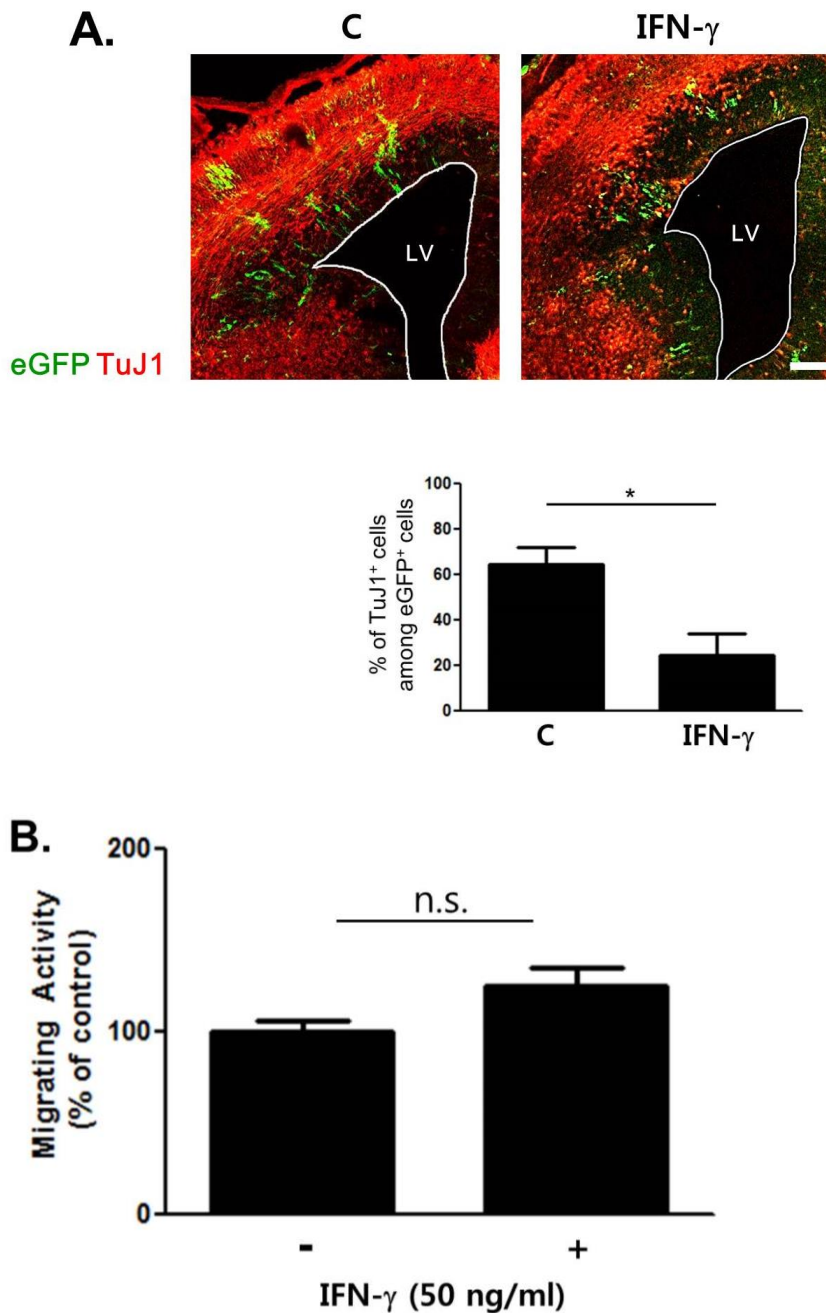
## **2.2. Effects of IFN- $\gamma$ on the neuronal differentiation of NPCs *in vivo***

To confirm the inhibitory effect of IFN- $\gamma$  on neuronal differentiation *in vivo*, a retroviral vector, expressing IFN- $\gamma$  and eGFP from a bicistronic message, was injected into the ventricles of E9.5 embryonic brains using the UIGD technique [120]. As described in the previous chapter, when NPCs in the VZ are transduced with such retroviral vectors, the neurons produced from the NPCs are supposed to migrate away from the VZ during neurogenesis. The brains were analyzed at E14.5, when neurogenesis reaches its peak level [121]. When embryonic brains were injected with a control vector expressing only eGFP, 64 $\pm$ 13% of eGFP-positive cells were localized in the TuJ1-stained region. By contrast, when the vector expressing both IFN- $\gamma$  and eGFP was delivered to embryonic brains, the transduced cells were predominantly found in the VZ (Fig.V-4A). Only approximately 24% of cells were positive for TuJ1, which was significantly lower than that of the control ( $P=0.0287$ ). These data suggest that IFN- $\gamma$  indeed suppresses neuronal differentiation during brain development.

To determine whether the retention of NPCs in the VZ in the IFN- $\gamma$  group resulted from the inhibition of cell migration, the effects of IFN- $\gamma$  on the migration of NPCs were also tested. Primary NPCs were allowed to form neurospheres and were allowed to differentiate with or without IFN- $\gamma$  for 1 day. To determine the migrating ability of NPCs, the distance from the edge of the neurosphere to the furthest outgrowing cell was measured and normalized to the diameter of the neurosphere (Fig. V-4B). There was no statistically significant difference between the control and the group treated with IFN- $\gamma$ , suggesting that IFN- $\gamma$  treatment does not affect the migrating ability of NPCs.



**Figure V-3. IFN- $\gamma$  does not affect the viability and the apoptosis of primary NPCs.** (A) Primary NPCs were differentiated with various concentrations of IFN- $\gamma$  for 2 days and subjected to the MTT assay. The percentages of viable cells compared with the control are shown. (B) Primary NPCs were differentiated with different concentrations of IFN- $\gamma$  for 2 days, and then the TUNEL assay was performed. DNase-treated cells were used as a positive control (PC). Representative images of the TUNEL staining and the percentage of TUNEL-positive cells are shown. DNA was labeled with Hoechst 33258 (blue). Scale bar, 50  $\mu$ m. The statistical significance of differences between two groups was analyzed using the unpaired *t* test. Other differences in values were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. \*\**P* < 0.01; \*\*\**P* < 0.001; n.s., not significant.



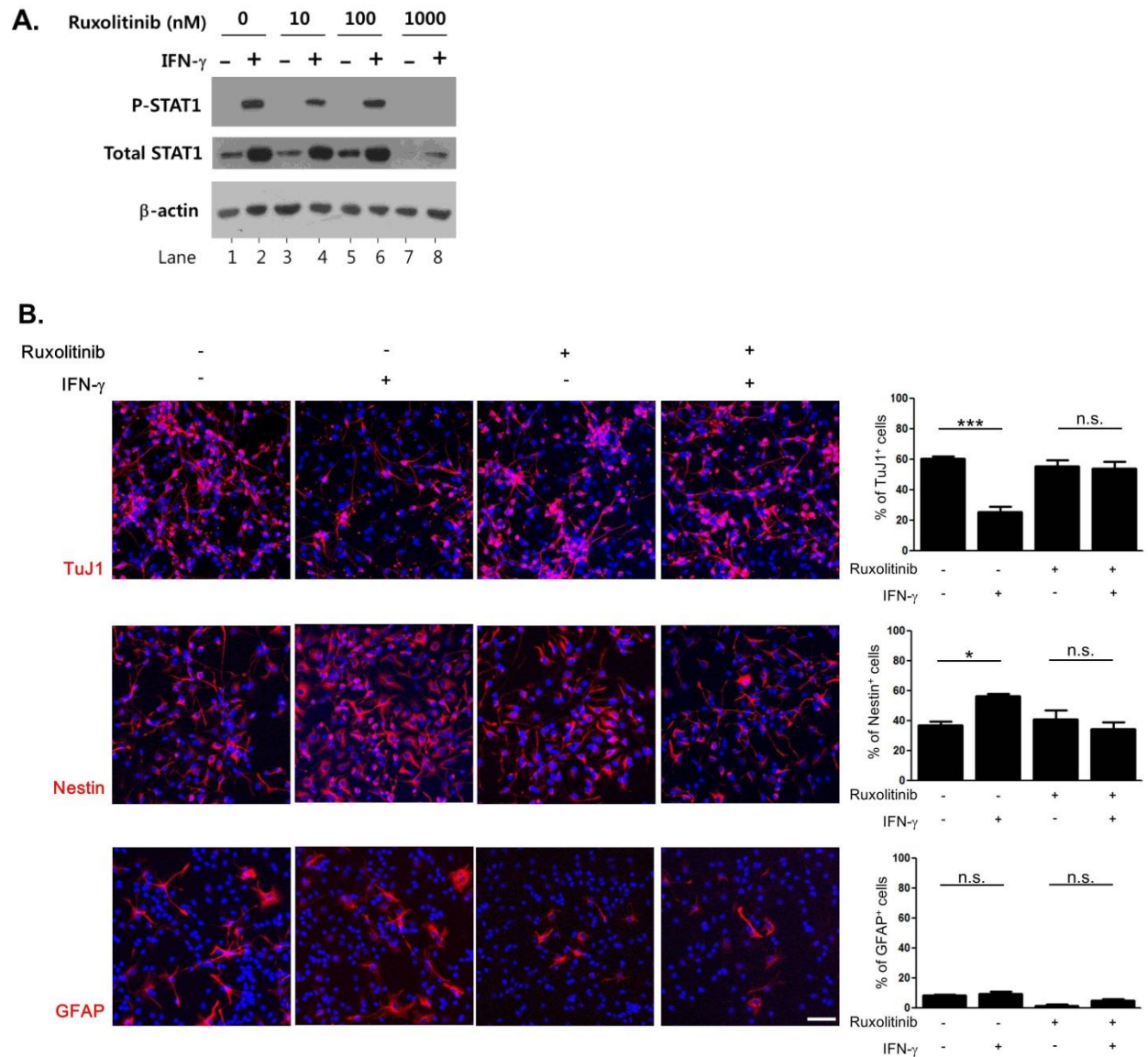
**Figure V-4. IFN- $\gamma$  inhibits neuronal differentiation *in vivo*.** (A) Retroviral vectors expressing IFN- $\gamma$  and eGFP were injected into E9.5 embryonic brains ( $n = 3/\text{group}$ ) and then the brains were immunostained with antibodies to eGFP and TuJ1 at E14.5. Representative images of sections and the percentages of TuJ1-positive neurons among GFP-positive cells are shown. (B) Primary neurospheres were differentiated with 50 ng/mL IFN- $\gamma$  for 1 day. Migrating activity was calculated by measuring the distance from the edge of the neurosphere to the furthest outgrowing cell and the diameter of the neurospheres. Scale bar, 100  $\mu\text{m}$ . The statistical significance of differences was analyzed using the unpaired  $t$  test. \* $P < 0.05$ . LV, lateral ventricle; C, control.

### **2.3. Involvement of the JAK/STAT1 pathway in the IFN- $\gamma$ -mediated inhibition of neurogenesis**

To understand the downstream mechanism of the inhibitory role of IFN- $\gamma$  in embryonic neurogenesis, the involvement of the JAK/STAT1 pathway was analyzed as it is the main signaling route activated by IFN- $\gamma$ . Primary E14.5 NPCs were differentiated with DMEM containing 2% FBS and treated with IFN- $\gamma$  alone or in combination with 1  $\mu$ M JAK1/2 inhibitor (Ruxolitinib) for 2 days. The activity of Ruxolitinib was confirmed by examining the phosphorylation level of STAT1 by western blotting. Upon IFN- $\gamma$  treatment, the STAT1 tyrosine residue was phosphorylated as expected, but phosphorylation was totally inhibited when 1  $\mu$ M Ruxolitinib was added. The level of total STAT1 was also decreased upon 1  $\mu$ M Ruxolitinib treatment. Since Ruxolitinib is a JAK1/2 inhibitor, proteins other than STAT1 that are activated by JAK1/2 could have affected the level of total STAT1 (Fig.V-5A). The proportion of TuJ1-positive cells was reduced from  $60\pm 3.1\%$  to  $26\pm 5.6\%$  upon IFN- $\gamma$  treatment and was restored to the control level when Ruxolitinib was added concurrently. Conversely, the proportion of Nestin-positive cells increased from  $36\pm 4.8\%$  to  $56\pm 2.9\%$  after IFN- $\gamma$  treatment but remained similar to the control level after treatment with both IFN- $\gamma$  and Ruxolitinib ( $41\pm 11\%$  and  $34\pm 8.1\%$ , respectively). There was no significant difference in the number of GFAP-positive astrocytes between the control and IFN- $\gamma$ -treated groups (Fig.V-5B).

As JAK1/2 is involved in the activation of not only STAT1 but also other signaling pathways such as STAT3 and MAPK pathways [122-124], shRNA targeting STAT1 was employed to confirm the role of STAT1 and rule out the effects of other factors on neurogenesis. Retroviral vectors expressing three different shRNA sequences against STAT1, together with HA-tagged DsRed, were constructed. To determine the knock-down efficiency, NIH3T3 cells were transduced with retroviral vectors expressing control or STAT1 shRNA. Two days later, the level of STAT1 was measured by western blotting. STAT1 shRNA sequence #2 showed the strongest inhibition of STAT1 expression (approximately 67.2%) (Fig. V-6A), and therefore was chosen for subsequent experiments. NPCs were transduced with the retroviral vector expressing STAT1 shRNA and, 2 days later, induced to





**Figure V-5. Inhibition of the JAK/STAT1 pathway abrogated the IFN- $\gamma$ -mediated inhibition of neurogenesis. (A)** NPCs were induced to differentiate in the presence of different concentrations of Ruxolitinib for 12 hr. Proteins were prepared and subjected to Western blot using antibodies to P-STAT1 (Tyr701), total STAT1 and  $\beta$ -actin. **(B)** NPCs were induced to differentiate in the presence of 50 ng/mL IFN- $\gamma$  with or without 1  $\mu$ M Ruxolitinib for 2 days and then immunostained for TuJ1, Nestin, and GFAP. Representative images of the staining and the percentages of each cell type are shown.

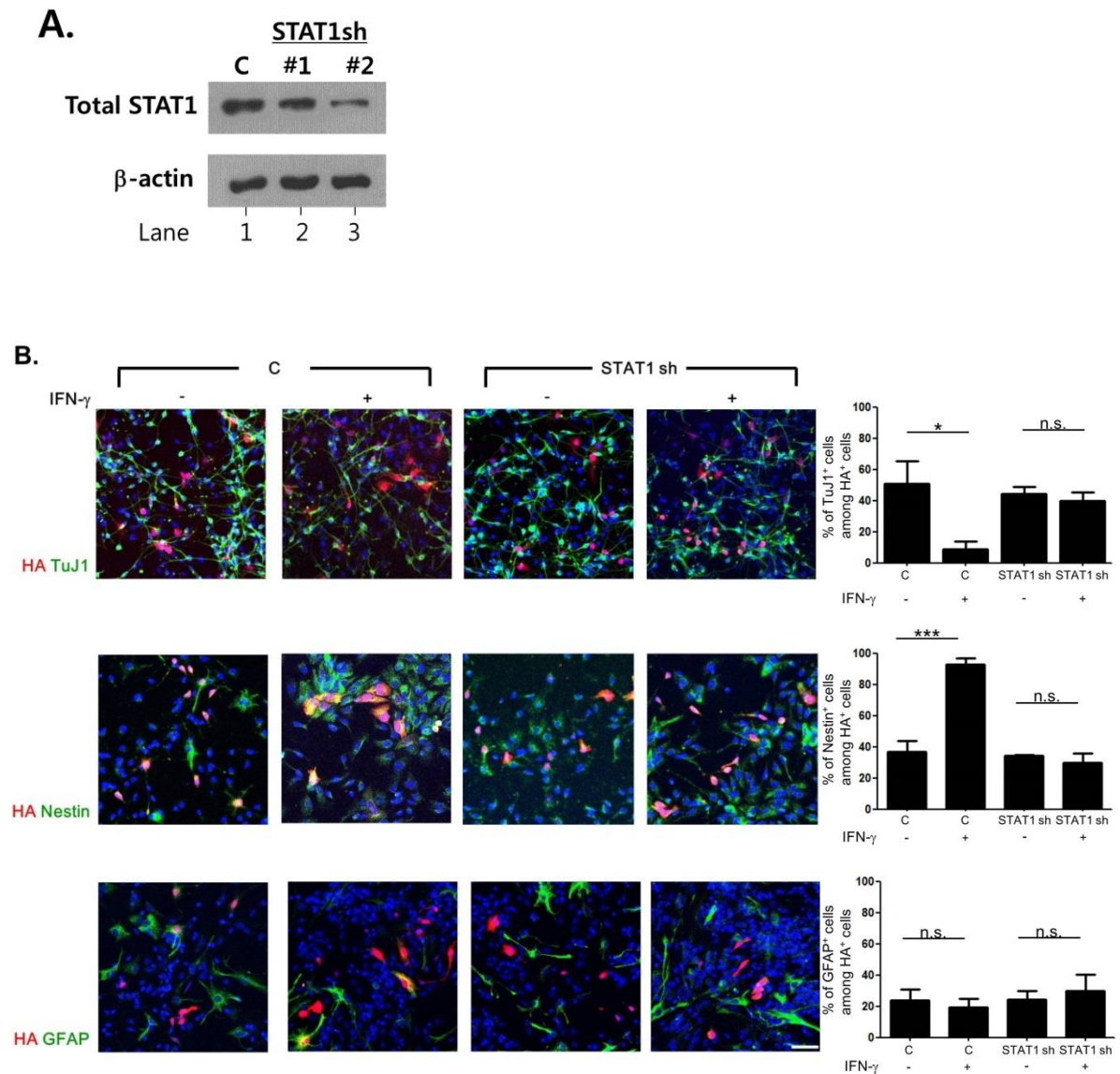
differentiate in the presence of IFN- $\gamma$  for 2 days. The percentages of HA-positive cells expressing different cell markers were calculated. Among the NPCs transduced with the control vector, the percentage of TuJ1-positive cells was reduced from  $51\pm 25\%$  to  $10\pm 8.4\%$  upon IFN- $\gamma$  treatment. When cells were transduced with the vector expressing STAT1 shRNA, however, there was no statistically significant difference between the control and IFN- $\gamma$ -treated groups ( $44\pm 7.9\%$  and  $40\pm 10\%$ , respectively). The percentage of Nestin-positive cells was increased approximately 2.5-fold upon IFN- $\gamma$  treatment, while such an increase was not observed in cells expressing STAT1 shRNA. Neither IFN- $\gamma$  treatment nor the knock-down of STAT1 expression affected the number of GFAP-positive cells (Fig. V-6B). These results clearly show that the inhibition of neurogenesis by IFN- $\gamma$  is dependent on the JAK/STAT1 signaling pathway.

## 2.4. Effects of IFN- $\gamma$ on the expression of *Neurogenin 2*

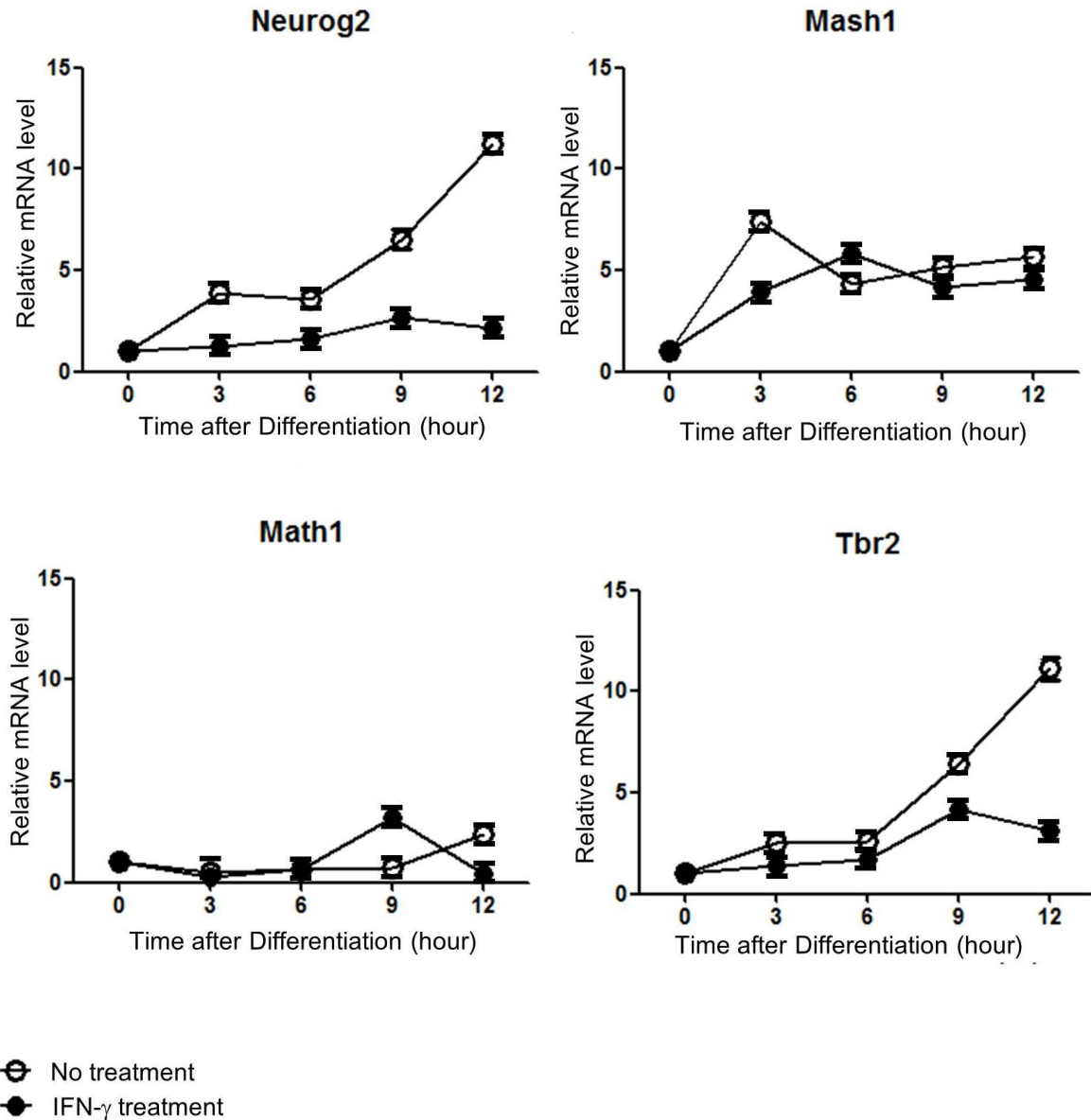
It was tested whether IFN- $\gamma$  had an effect on the expression of the bHLH proneural genes as they are key players in the neuronal differentiation of NPCs [125]. Primary NPCs were treated with IFN- $\gamma$  and differentiated with DMEM containing 2% FBS. Total RNA was collected every 3 hr up to 12 hr, and the RNA levels of *Neurog2*, *Mash1* and *Math1* were analyzed by qRT-PCR. While the expression levels of *Mash1* and *Math1* were similar between the control and IFN- $\gamma$ -treated groups, there was a remarkable change in the case of *Neurog2*. The RNA level of *Neurog2* was induced more than 10-fold upon differentiation, while this induction was almost completely inhibited in the IFN- $\gamma$ -treated group (Fig. V-7). To determine whether an increased level of *Neurog2* expression can generate actual biological effects, the expression level of *Tbr2*, one of the downstream targets of *Neurog2*, was measured [126]. The RNA level of *Tbr2* was also increased in a time-dependent manner upon differentiation, while its upregulation was reduced upon treatment with IFN- $\gamma$ , correlating with the level of *Neurog2* expression.

To investigate whether *Neurog2* is indeed the key downstream target of IFN- $\gamma$ , NPCs were transduced with a retroviral vector expressing *Neurog2* and eGFP from a bicistronic message for 2





**Figure V-6. IFN- $\gamma$ -mediated inhibition of neurogenesis depends on the JAK/STAT1 pathway.** (A) NIH3T3 cells were transduced with the same titer of retroviral vectors expressing control or STAT1 shRNA. After 2 days, proteins were prepared and subjected to Western blot using antibodies to total STAT1 and  $\beta$ -actin. (B) Primary NPCs were transduced with retroviral vectors expressing control or STAT1 shRNA together with HA-tagged DsRed. After 2 days, cells were differentiated with or without IFN- $\gamma$  and then co-immunostained for HA and different cell markers (TuJ1, Nestin, and GFAP). Representative images of the staining and the percentages of each cell type among HA-positive cells are shown. DNA was labeled with Hoechst 33258 (blue). Scale bar, 50  $\mu$ m. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. \* $P < 0.05$ ; \*\*\* $P < 0.001$ ; n.s., not significant. C, control.



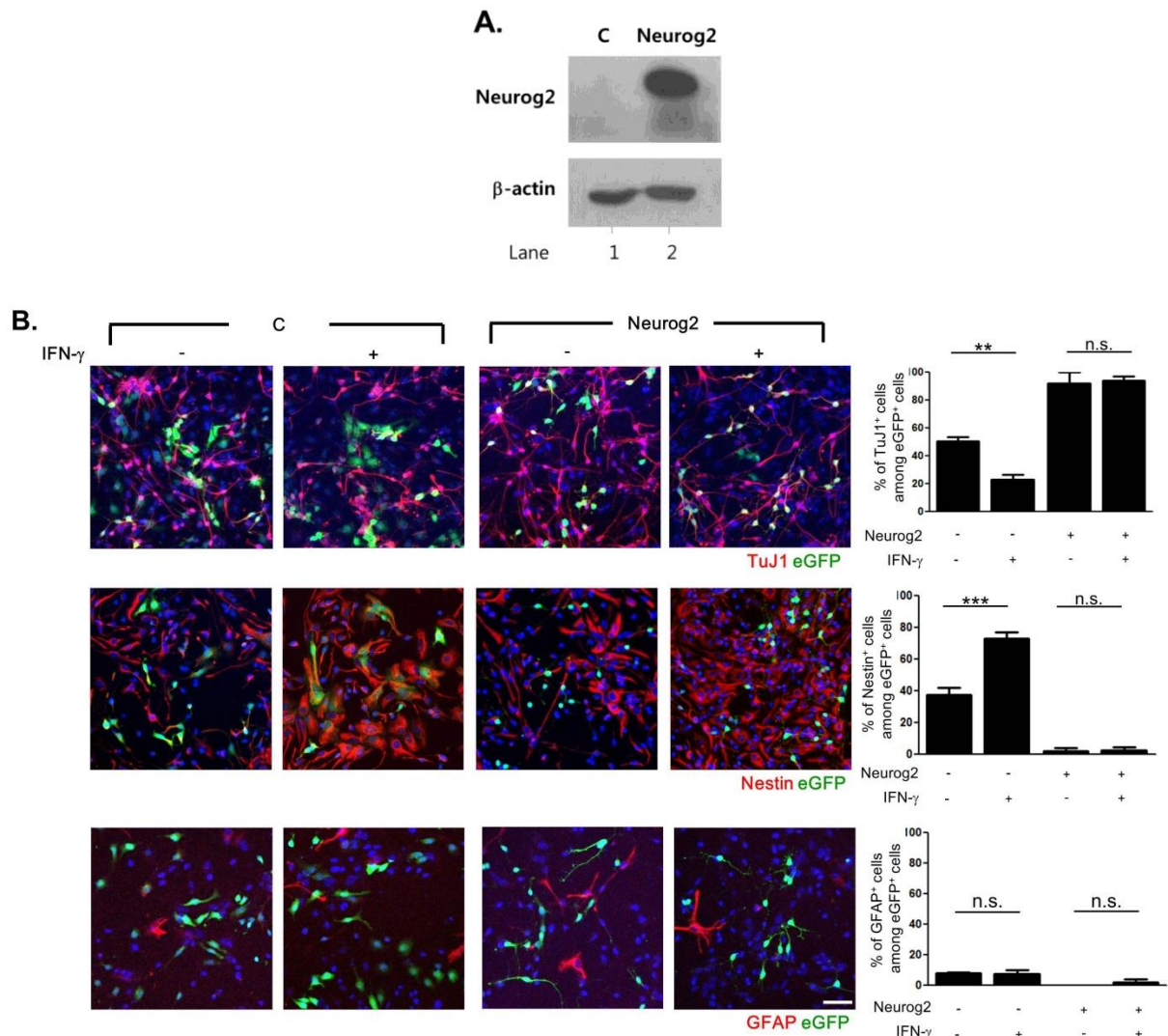
**Figure V-7. IFN- $\gamma$  negatively regulates the expression of *Neurog2* mRNA.** Total RNA was isolated from differentiated NPCs treated with or without 50 ng/mL IFN- $\gamma$  every 3 hr for 12 hr. The levels of *Neurog2*, *Math1*, *Mash1*, and *Tbr2* mRNA were measured by qRT-PCR. Differences between the values of two groups were analyzed by two-way ANOVA (Source of Variation = IFN- $\gamma$  treatment). P values for *Neurog2*, *Math1*, *Mash1*, and *Tbr2* are <0.0001, 0.3012, 0.3357, and 0.0001, respectively.

days and then differentiated with or without 50 ng/mL IFN- $\gamma$  for another 2 days. The expression of exogenous *Neurog2* was confirmed in NIH3T3 cells by western blotting (Fig. V-8A). IFN- $\gamma$  treatment did not affect the level of eGFP expression when NPCs were transduced with a control vector expressing eGFP only, suggesting that IFN- $\gamma$  does not have an influence on the function of viral long terminal repeats (data not shown). As expected, among the cells transduced with the control vector, IFN- $\gamma$  treatment reduced the number of TuJ1-positive cells by nearly half while increasing the number of Nestin-positive cells more than 2-fold. However, almost all cells overexpressing *Neurog2* had differentiated to TuJ1-positive neurons, and IFN- $\gamma$  treatment showed no inhibitory effect ( $92\pm 14\%$  and  $94\pm 5.2\%$ , respectively). There was no statistically significant difference in the percentage of GFAP-positive cells between the control and IFN- $\gamma$ -treated groups (Fig. V-8B). These results reveal that the inhibition of *Neurog2* expression is necessary for the IFN- $\gamma$ -mediated inhibition of neurogenesis.

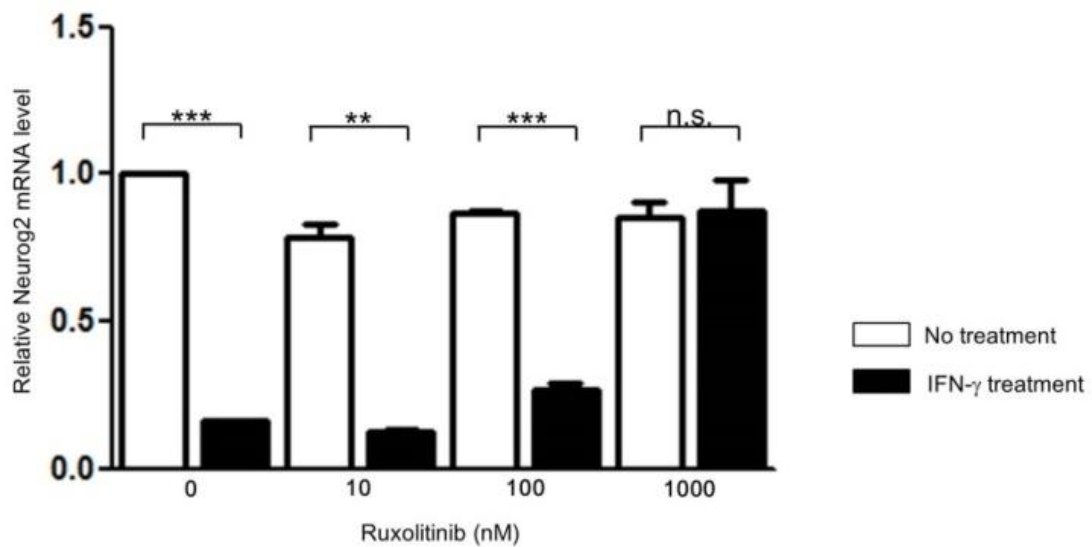
Since the effects of IFN- $\gamma$  on neurogenesis depend on the JAK/STAT1 pathway, it was tested whether inhibition of the JAK/STAT1 pathway would affect the IFN- $\gamma$ -mediated downregulation of *Neurog2* expression. NPCs were treated with IFN- $\gamma$  in the presence of 10 nM to 1  $\mu$ M Ruxolitinib and differentiated for 12 hr. Total RNA was isolated, and the level of *Neurog2* expression was determined by qRT-PCR (Fig. V-9). IFN- $\gamma$  treatment reduced the expression of *Neurog2* by approximately 84%, but the expression level was restored to that of the control at 1  $\mu$ M Ruxolitinib. Taken together, these data indicate that IFN- $\gamma$  inhibits neurogenesis by inhibiting *Neurog2* expression through the JAK/STAT1 pathway.

### 3. Discussion

The role of IFN- $\gamma$  in neurogenesis has been controversial. The results reported in this chapter demonstrate that IFN- $\gamma$  negatively regulates neuronal differentiation both *in vitro* and *in vivo*. However, these data are in contrast with the results of some previous studies. For example, a study involving the C17.2 cell line has showed that IFN- $\gamma$  induces neuronal differentiation by activating the JNK pathway [116]. In the study of Walter *et al.*, treatment of proliferating E14 neural stem/precursor



**Figure V-8. Overexpression of *Neurog2* abrogated the IFN- $\gamma$ -mediated negative regulation of neurogenesis.** (A) NIH3T3 cells were transduced with a retroviral vector expressing *Neurog2*. After 2 days, cell lysates were prepared and subjected to Western blot using antibodies to *Neurog2* and  $\beta$ -actin. (B) Primary NPCs were transduced with a retroviral vector expressing *Neurog2* and eGFP for 2 days. Cells were then differentiated with or without IFN- $\gamma$  for another 2 days and subjected to co-immunostaining for eGFP and TuJ1, Nestin, or GFAP. Representative images and the percentage of each cell type among the eGFP-positive cells are shown. DNA was labeled with Hoechst 33258 (blue). Scale bar, 50  $\mu$ m. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. \*\*P < 0.01; \*\*\*P < 0.001; n.s., not significant.



**Figure V-9. IFN- $\gamma$ -mediated downregulation of *Neurog2* expression is dependent on the JAK/STAT1 pathway.** Primary NPCs were differentiated in the presence of different concentrations of Ruxolitinib with or without 50 ng/mL IFN- $\gamma$ . After 12 hr, RNA was isolated and the level of *Neurog2* mRNA was measured by qRT-PCR. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; n.s., not significant.

cells with 1,000 U/mL IFN- $\gamma$  (equivalent to 1 mg/mL) resulted in atypical gene expression and cell functions through regulation of the Shh pathway [127]. None of the above observations were made in this study. The different findings obtained in these studies may be explained by the various types of cells and the different concentrations of IFN- $\gamma$  used, or they may be due to differences in other experimental conditions such as the time of NPC isolation and the availability of cofactors involved in the JAK/STAT1 pathway.

Many downstream targets of IFN- $\gamma$  are known, but they have not been extensively studied in the context of embryonic neuronal differentiation. According to the results obtained from this chapter, one of the final target genes of IFN- $\gamma$  appears to be *Neurog2*. *Neurog2* is an essential factor during the initiation of the neuronal differentiation of NPCs and it is sufficient to generate neurons from mouse embryonic stem cells [128-131]. Thus, *Neurog2* is a key factor in the determination of neural cell types and its expression pattern is tightly regulated, but only a few factors are known to regulate its expression [132-134]. In the present study, IFN- $\gamma$  specifically suppressed the RNA level of *Neurog2* among many proneural genes. IFN- $\gamma$  treatment inhibited the upregulation of *Neurog2* expression in NPCs throughout the differentiation period, and the overexpression of *Neurog2* completely abrogated the inhibitory effect of IFN- $\gamma$  on neurogenesis. Because STAT1 can regulate gene expression in various ways, there are many possible mechanisms by which it might control the level of *Neurog2* mRNA. Further investigations are required to unravel the precise mechanism of how the JAK/STAT1 pathway controls the expression level of *Neurog2* mRNA.

Several recent studies have suggested that the cause of neurological disorders in infants that survive congenital infections is the inflammatory response, rather than the pathogen itself [69]. Lipopolysaccharide or polyinosinic:polycytidylic acid (synthetic double-stranded RNA) injections administered to rat or mouse dams resulted in sensorimotor gating dysfunction, increased anxiety, impairment of social interactions, and other abnormal behaviors in newborns, showing that an inflammatory response is sufficient to cause behavioral abnormalities associated with brain dysfunction [135-137]. IFN- $\gamma$  is one of the cytokines that is known to be released in the fetal brain during infection [109, 111, 138]. As *Neurog2* is known to generate glutamatergic neurons while

suppressing the generation of GABAergic neurons, the downregulation of *Neurog2* expression due to IFN- $\gamma$  may lead to an abnormal ratio of glutamatergic to GABAergic neurons in the mature brain. An imbalance of glutamate and GABA has been reported to be a major cause of various neurological diseases including autism, Rett syndrome, schizophrenia, and mood disorders [139-141]. In this regard, it is interesting to note that the glutamate to GABA ratio was reduced whereas the level of IFN- $\gamma$  was increased in the plasma of autistic patients compared with healthy subjects, suggesting a link between IFN- $\gamma$  and brain abnormalities [139]. Although it is not yet clear what the consequences of the IFN- $\gamma$ -mediated dysregulated neuronal differentiation during embryonic development are, the findings from this chapter may provide an interesting starting point for understanding the role(s) of IFN- $\gamma$  during brain development, and ultimately, the neurodevelopmental disorders caused by congenital infections.

## **CHAPTER VI**

# **Search for Cis-acting Sequence Involved in the IFN- $\gamma$ -mediated Inhibition of *Neurog2* Expression**



# 1. Background

*Neurog2*, a bHLH transcription factor, is an atonal proneural gene, which is expressed in the brain and spinal cord throughout development. Its expression is restricted to the dorsal telencephalon in the brain and it is known to play a role in the differentiation of early-born neurons in the cerebral cortex [128, 129, 142, 143]. It is an essential factor for initiating the neuronal differentiation of NPCs, and it is sufficient to generate neurons from mouse embryonic stem cells [130-132]. Although *Neurog2* has been known as a key factor in the determination of neural cell types, and its expression pattern is tightly regulated, only a few factors are known to regulate its expression.

The regulation of proneural genes occurs mostly at the transcriptional level. The transcription factors that are known to regulate *Neurog2* expression include the paired-box transcriptional activators, Pax3 and Pax6, and the transcriptional repressor, Hes1. Pax3 has been studied widely in the context of cell migration, neurogenesis, cardiogenesis, and melanocyte stem cell differentiation. Pax3 mutant mice exhibit premature neurogenesis with abnormal expression of *Hes1* and *Neurog2* [133, 144]. By binding to the promoters of *Hes1* and *Neurog2*, Pax3 activates the expression of *Hes1* during the early stages of neurogenesis to promote the proliferation of NPCs, and then activates *Neurog2* at a later stage to initiate neuronal differentiation. Another homeodomain protein, Pax6, plays a key role in patterning the neural tube in response to Shh, and it is expressed in the spinal cord and lateral cortex. Pax6 activates *Neurog2* expression by binding directly to the E1 enhancer element [145]. *Hes1*, a Notch target gene, is expressed in NPCs in an oscillating pattern with *Neurog2* in the developing telencephalon [146, 147]. The expression of *Neurog2* leads to Notch ligand expression, which activates the Notch signaling pathway in neighboring cells, producing Hes1. Hes1 then inactivates *Neurog2* via direct promoter binding, blocking its transcription. FGF-2 has also been reported to negatively regulate *Neurog2* expression by activating the Notch pathway in the dorsal root ganglia [148].

Recently, a transcription factor, RP58 (ZNF238), has also been implicated in the activation of *Neurog2*. RP58 is highly conserved (>95%) in humans and mice, and it is expressed in NPCs and

neurons. The neural-specific RP58 KO mice show defects in brain development with decreased neurogenesis, suggesting a role for RP58 in early corticogenesis. RP58 binds directly to the promoter region of *Neurog2* and represses its expression in differentiating neurons. It also represses the expression of *NeuroD1* in INPs, controlling the timing and the extent of neuron generation [149]. The key player in the Wnt pathway,  $\beta$ -catenin, has also been found to increase the *Neurog2* promoter activity in 293 cells, although the direct binding site has not yet been experimentally determined [150].

The expression of *Neurog2* is known to be regulated primarily at its transcription level. However, no definite link has been suggested between the inflammatory signaling pathway and the regulation of *Neurog2*. In this chapter, the mechanism by which IFN- $\gamma$  inhibits *Neurog2* transcription was investigated using a reporter plasmid under the control of the *Neurog2* promoter. The promoter activity was tested in various experimental settings using recombinant IFN- $\gamma$  and a cell line overexpressing STAT1 by employing a transient or stable transfection method. In every case, the activation of STAT1 led to the reduction in the *Neurog2* promoter activity. The data from the experiments involving a translational inhibitor and different forms of the *Neurog2* promoter suggested that the IFN- $\gamma$ -mediated negative regulation of *Neurog2* might be achieved by complex mechanisms, which may require *de novo* protein synthesis, rather than by simple promoter binding.

## 2. Results

### 2.1 Sequence Analysis of the *Neurog2* Promoter

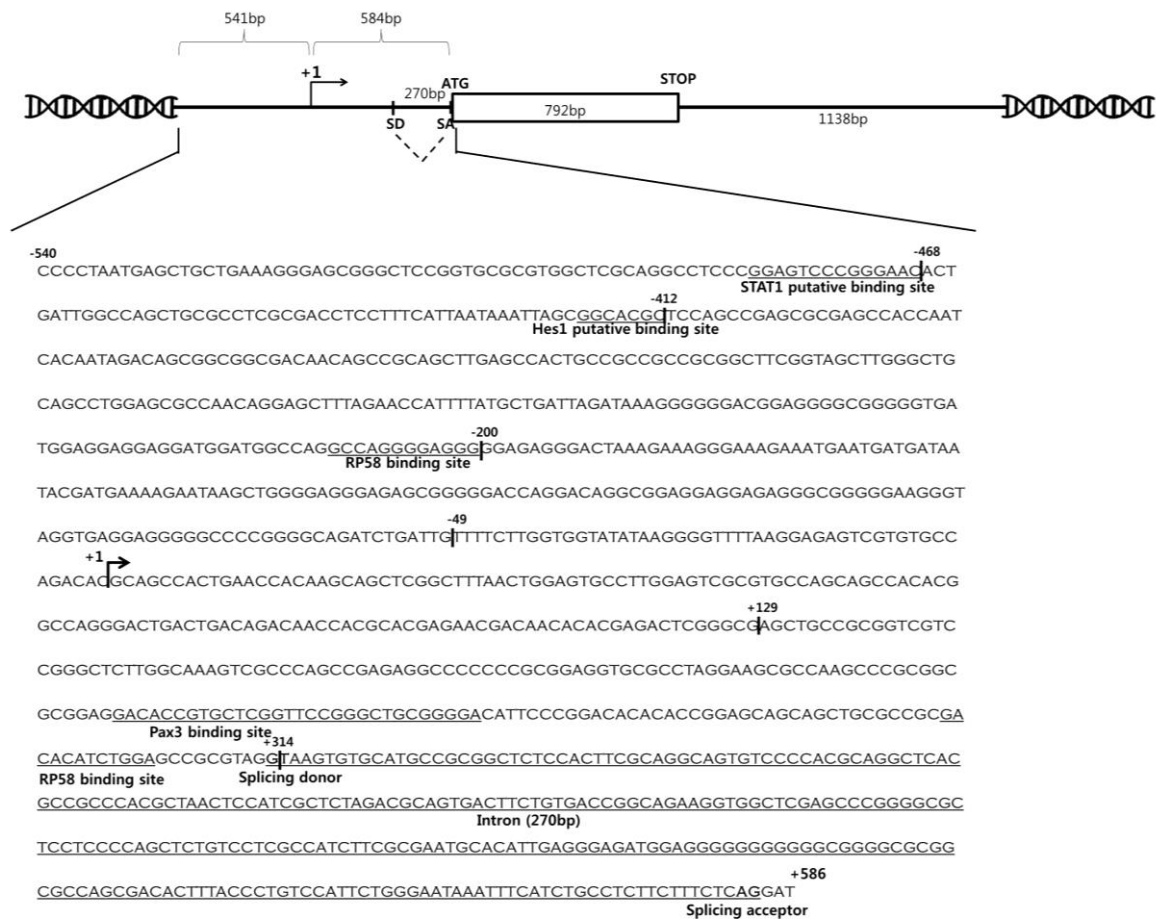
The *Neurog2* genome comprises a 5' untranslated region (UTR) including an intron, *Neurog2* cDNA, and a 3' UTR. *Neurog2* mRNA is spliced at splicing donor and splicing acceptor sites, which are located at +314 and +581, respectively. The STAT1 transcription factor can bind directly to a specific DNA sequence known as the IFN- $\gamma$  activating sequence (GAS); therefore, the *Neurog2* promoter was analyzed using the TRANSFAC database to determine whether it contains a functional GAS. One putative STAT1 binding site (5'-ggagtcccgggaa-3') was found 468 bp upstream from the

transcription start site. To investigate whether the IFN- $\gamma$ -mediated negative regulation of *Neurog2* expression occurs at the level of transcriptional initiation, the minimal essential *Neurog2* promoter containing the 5' upstream region of the *Neurog2* gene from -540 to +586 bp was cloned (Fig. VI-1) [133].

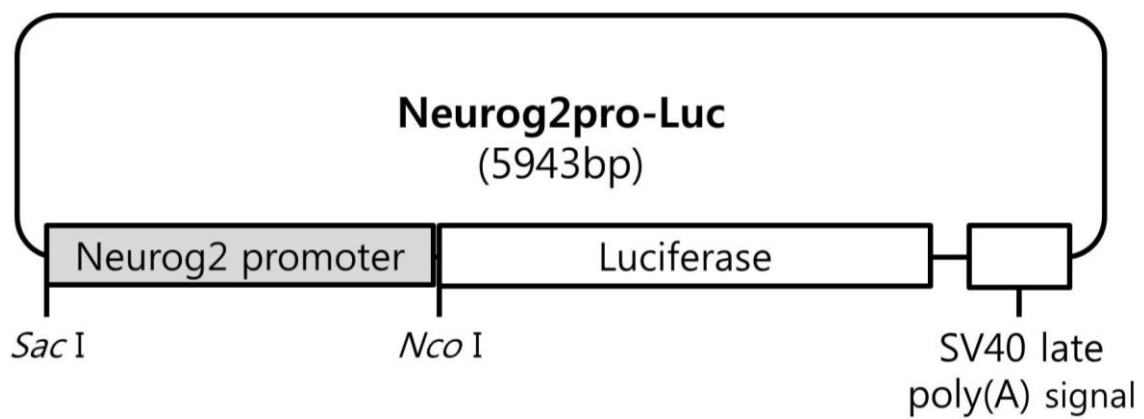
## **2.2 Effects of IFN- $\gamma$ on the activity of the transiently transfected *Neurog2* promoter**

To perform a promoter reporter assay, a luciferase expression plasmid under the control of the *Neurog2* promoter (Neurog2pro-Luc) was constructed (Fig. VI-2). First, primary NPCs were electroporated with the Neurog2pro-Luc and after 2 days, they were differentiated with 50 ng/mL IFN- $\gamma$  for 12 hr. The level of luciferase activity increased almost two-fold upon differentiation and this upregulation was completely inhibited when IFN- $\gamma$  was added (Fig. VI-3A). When NPCs were treated with 10, 50, or 100 ng/mL IFN- $\gamma$ , the *Neurog2* promoter activity was decreased by 13.5%, 47.7%, and 59%, respectively, demonstrating a dose-dependent response (Fig. VI-3B). These results indicate that the *Neurog2* promoter activity is increased upon differentiation, while IFN- $\gamma$  inhibits this up-regulation at the promoter level.

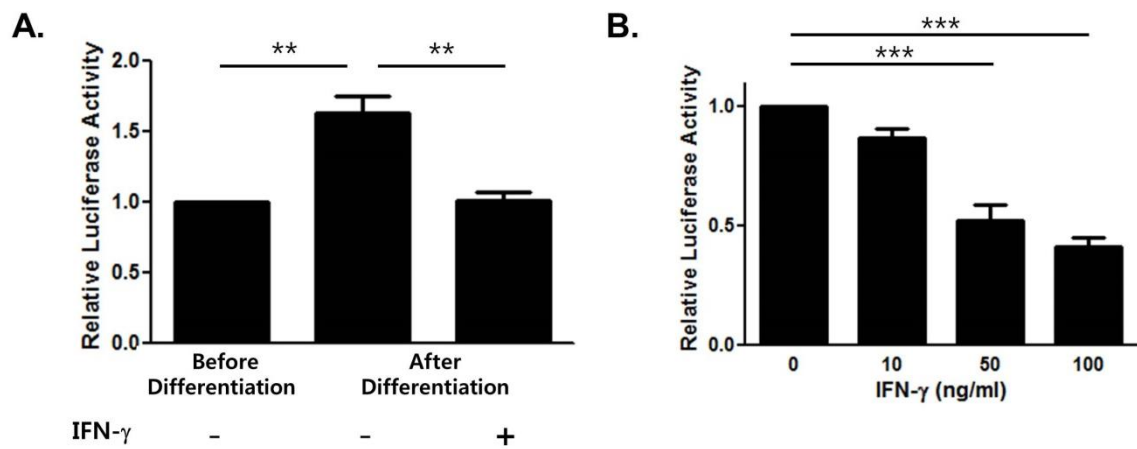
The effects of IFN- $\gamma$  treatment on the *Neurog2* promoter activity were also tested in NIH3T3 cells, which are commonly used in luciferase assays and known to be responsive to murine IFN- $\gamma$ . NIH3T3 cells were treated with IFN- $\gamma$  for 1 day, and the level of *Neurog2* mRNA was measured (Fig. VI-4A). Similar to the results obtained from primary NPCs, IFN- $\gamma$  treatment reduced *Neurog2* mRNA expression by approximately 60%. When NIH3T3 cells were transfected with Neurog2pro-Luc and treated with 10, 50, or 100 ng/mL IFN- $\gamma$  for 1 day, there was 25.1%, 35.3%, and 37% reduction in the promoter activity, respectively (Fig. VI-4B). This data show that the downregulation of *Neurog2* expression and the promoter activity upon IFN- $\gamma$  treatment in NIH3T3 cells were similar to the data observed in primary NPCs, validating the use of NIH3T3 cells in further experiments.



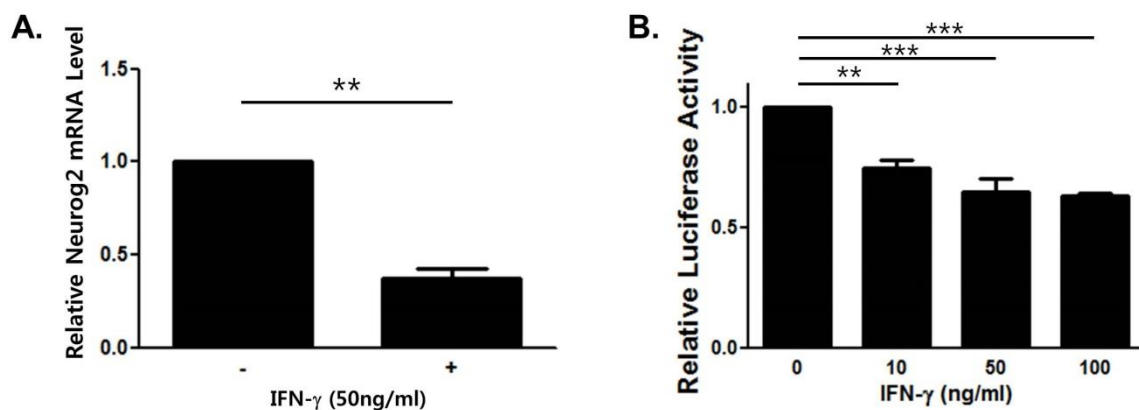
**Figure VI-1. *Neurog2* promoter sequence.** The *Neurog2* genome comprises of the promoter region (1125bp) including a 5' UTR with an intron, *Neurog2* cDNA (792 bp), and a 3' UTR (1138 bp). *Neurog2* mRNA is spliced at splicing donor site located at +314 and splicing acceptor site at +581. The region between 540 bp upstream and 586 bp downstream from the transcription start site (+1), which includes the 270bp intron is known as the minimal *Neurog2* promoter. Experimentally proven or putative binding sequences of different regulators in the *Neurog2* promoter are indicated. SD, splicing donor; SA, splicing acceptor.



**Figure VI-2. Schematic diagram of the Neurog2pro-Luc.** A *SacI/NcoI* fragment that lies between 540bp upstream and 586bp downstream from the *Neurog2* transcription start site was cloned into the pGL3-Basic plasmid (promega) using PCR. The diagram is not to scale.



**Figure VI-3. IFN- $\gamma$  downregulates the activity of the *Neurog2* promoter in primary NPCs. (A, B)** Primary NPCs were isolated from E14.5 embryonic brains and electroporated with pNeurog2-Luc. After 1 day, cells were differentiated with or without IFN- $\gamma$  for 12 hr and subjected to a luciferase reporter assay. The relative luciferase activity is shown. \*\*P < 0.01; \*\*\*P < 0.001.



**Figure VI-4. IFN- $\gamma$  downregulates the expression of *Neurog2* mRNA and the activity of the *Neurog2* promoter in NIH3T3 cells.** NIH3T3 cells were transfected with the pNeurog2-Luc. After 3 hr, IFN- $\gamma$  was added with fresh medium for 1 day. **(A)** Total RNA was isolated, and the level of *Neurog2* mRNA was measured by qRT-PCR. **(B)** Cell lysates were prepared and subjected to a luciferase reporter assay. The relative luciferase activity is shown. \*\*P < 0.01; \*\*\*P < 0.001.

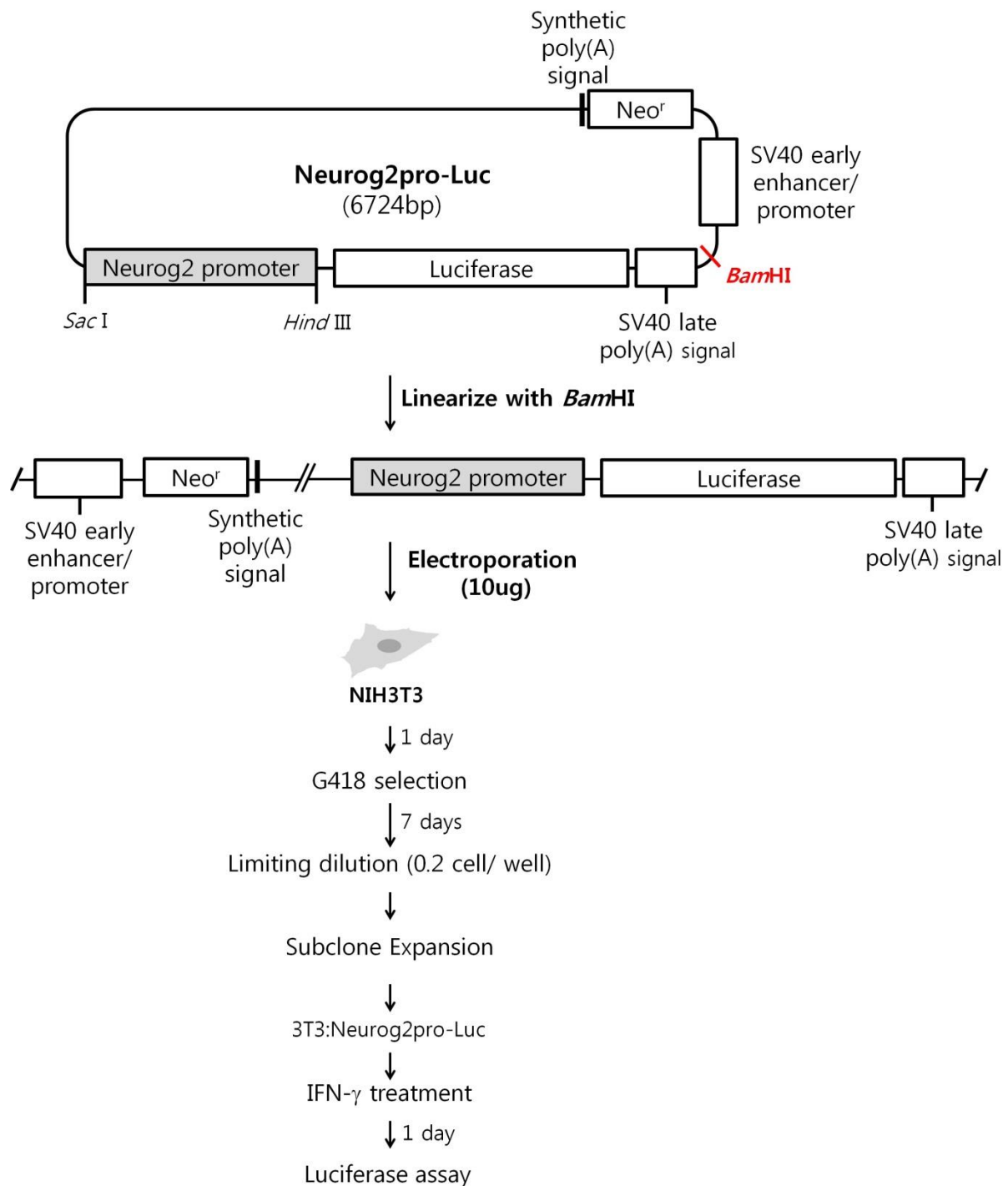
### **2.3 Effects of IFN- $\gamma$ on the activity of the stably transfected *Neurog2* promoter**

The results obtained using transiently transfected reporter plasmids could be fundamentally different from those observed in nature, because the gene is normally present in the chromosome as a single copy or very few copies, whereas multiple extrachromosomal copies of the promoter are present in the case of transient transfection. Therefore, the effects of IFN- $\gamma$  on a stably integrated *Neurog2* promoter were investigated. The Neurog2pro-Luc-Neo<sup>R</sup> plasmid that contains a neomycin-resistance gene was constructed and linearized at the *Bam*HI restriction site to facilitate the efficient integration of the plasmid DNA into the cellular chromosome (Fig. VI-5). The linearized Neurog2pro-Luc-Neo<sup>R</sup> plasmid was electroporated into NIH3T3 cells and subjected to G418 selection for 1 week. Using the serial dilution method, the cells were plated onto 96-well plates, and eight drug-resistant cell clones were obtained. The clone with the highest basal luciferase activity was selected, expanded, and designated as 3t3: Neurog2pro-Luc. The 3t3: Neurog2pro-Luc cells were treated with IFN- $\gamma$  for 1 day and subjected to a luciferase assay. IFN- $\gamma$  treatment reduced the *Neurog2* promoter activity by approximately 40% (Fig. VI-6). This result is in a good agreement with the data obtained from the transient transfection assay, suggesting that the transient transfection method is suitable for further experiments.

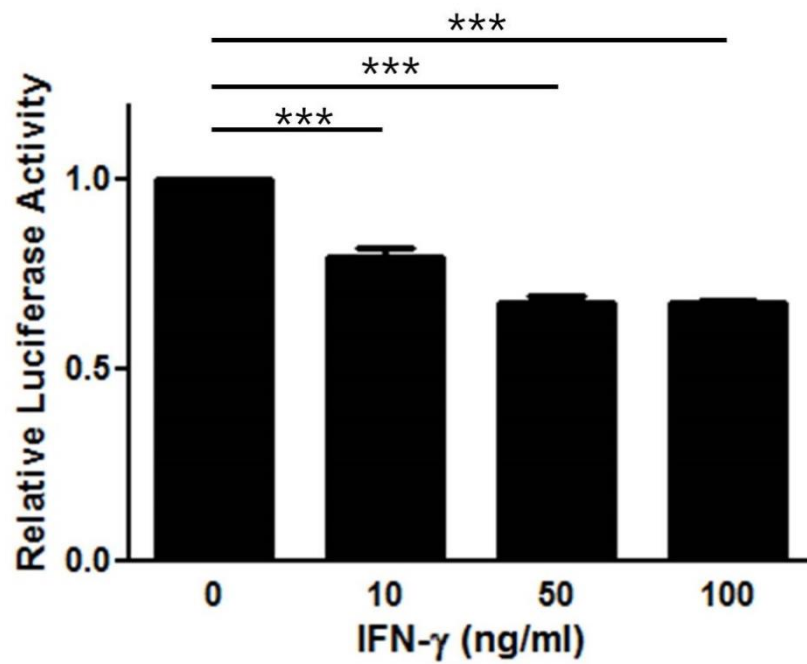
### **2.4 Effects of STAT1 overexpression on the activity of the *Neurog2* promoter**

The IFN- $\gamma$ -mediated downregulation of *Neurog2* expression is dependent on the JAK/STAT1 pathway; therefore, the effects of STAT1 expression on the *Neurog2* promoter activity were investigated. A cell line (3T3-STAT1ca) that overexpresses a constitutively active form of STAT1 (STAT1ca) was generated using a retroviral vector. STAT1ca contains two cysteine residues in the place of Ala-656 and Asn-658, which allows the formation of a dimer via a disulfide bond [18] (Fig.





**Figure VI-5. Overall procedure used for the stable transfection of the pNeurog2-Luc plasmid.** A *Sac*I/*Hind*III fragment that lies between 540 bp upstream and 586 bp downstream from the transcription start site was cloned into pGL4.17 plasmid using PCR and designated as Neurog2pro-Luc-Neo<sup>R</sup>. The plasmid was linearized with *Bam*HI. 10  $\mu$ g of the linearized plasmid was electroporated into NIH3T3 cells followed by G418 selection. The G418-resistant cell clone with the highest basal luciferase activity was selected, expanded, and designated as 3T3:Neurog2pro-Luc. The diagram is not to scale.



**Figure VI-6. Effects of IFN-γ on the expression of the integrated luciferase gene.** The selected NIH3T3 cells stably expressing luciferase gene under the control of the *Neurog2* promoter (3T3:Neurog2pro-Luc) were treated with different concentrations of IFN-γ for 1 day and subjected to a luciferase assay. The relative luciferase activity is shown. \*\*\*P < 0.001.

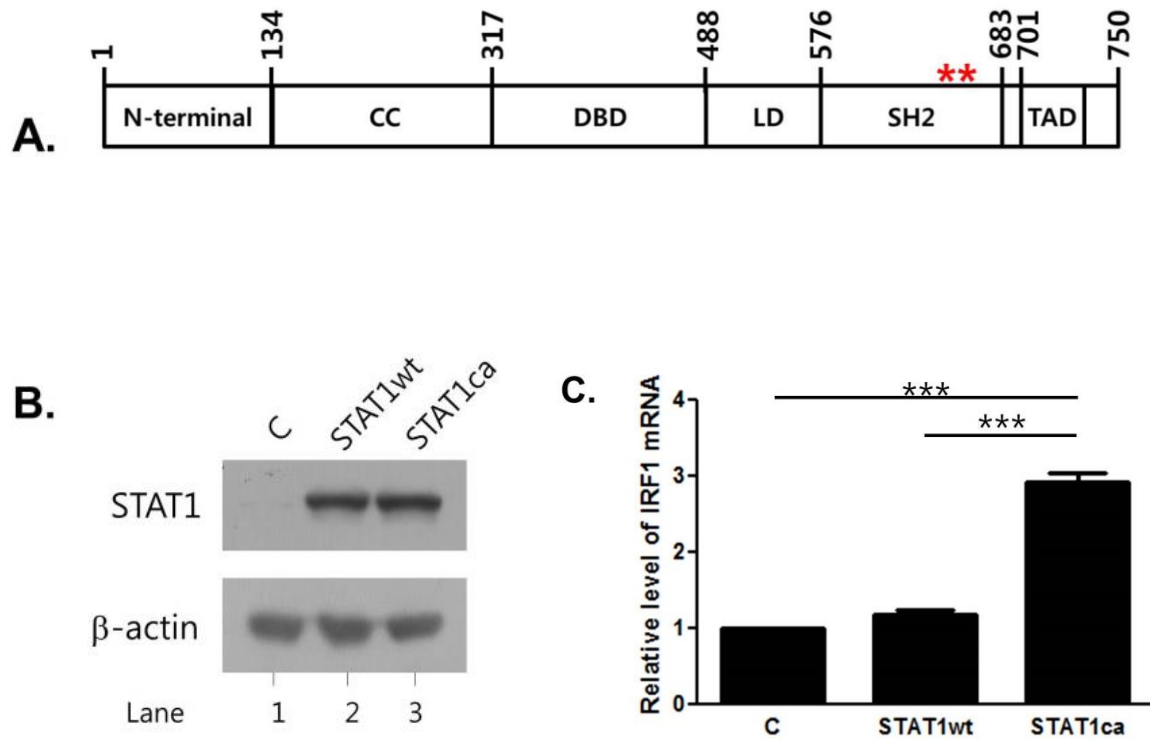
VI-7A). The expression and functionality of STAT1ca were confirmed by western blotting, and qRT-PCR that measured the mRNA levels of one of the STAT1 target genes, *Interferon regulatory factor 1* (*IRF1*) (Fig. VI-7B, C). STAT1 was not expressed in the control cells, whereas it was detected in the wild-type STAT1-expressing cells (3T3-STAT1wt) and 3T3-STAT1ca. Compared with the control and 3T3-STAT1wt, the level of *IRF1* mRNA in the 3T3-STAT1ca was approximately three times higher, indicating that STAT1ca is indeed constitutively active and able to activate downstream target genes. The *Neurog2*pro-Luc plasmid was transfected into the 3T3-control or -STAT1ca cells for 1 day, and the total proteins were extracted and subjected to a luciferase assay (Fig. VI-8A). The expression of STAT1ca did not affect the level of luciferase activity in promoter-less (mock) vector-transfected cells. Similar to the experiment performed with IFN- $\gamma$ , the luciferase activity level in the 3T3-STAT1ca was approximately half of that in the control cells, suggesting that STAT1 may play a key role in the IFN- $\gamma$ -mediated downregulation of *Neurog2* promoter activity. An IFN- $\gamma$  overexpressing cell line was also tested, however the stable expression of IFN- $\gamma$  affected the level of luciferase activity in mock vector-transfected cells, thus excluded for the further experiments (Fig. VI-8B).

## 2.5 Search for the IFN- $\gamma$ -responsive regulatory element in the *Neurog2*

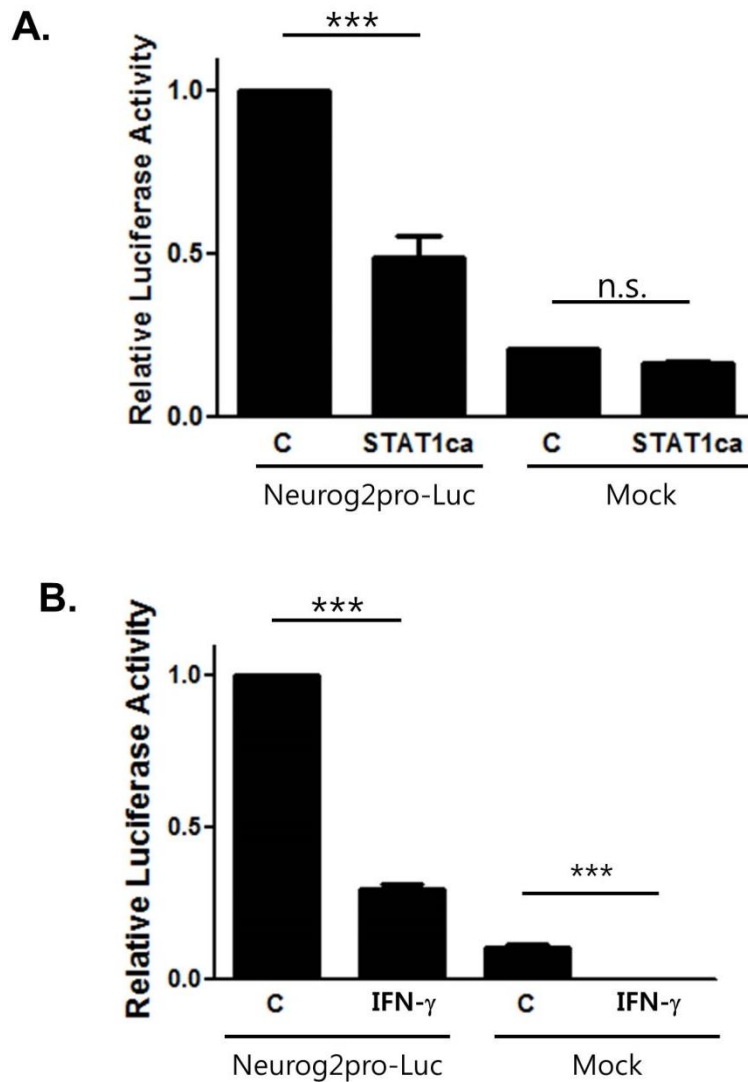
### Promoter

Eight different promoters with deletion or mutation were constructed to identify the IFN- $\gamma$ -responsive regulatory element in the *Neurog2* promoter (Fig. VI-9). Along with one promoter that lacks the putative STAT1 binding site (Fig. VI-9 #2), six other short forms of the *Neurog2* promoter were constructed as STAT1 is known to be permissive for mismatches and can bind to unique DNA sequences by interacting with other proteins [151]. A mutant form containing an inactive splicing site (Fig. VI-9 #8) was also constructed to determine whether the splicing event is crucial for *Neurog2* expression.

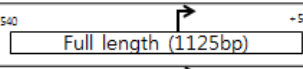
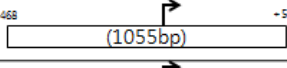
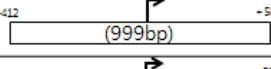
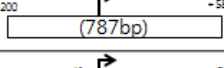
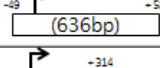
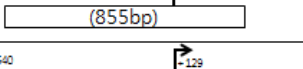
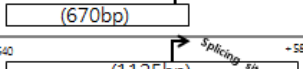
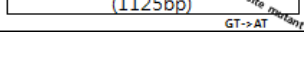
The 3T3-control or 3T3-STAT1ca cells were transfected with the appropriate luciferase



**Figure VI-7. Construction of a constitutively active form of STAT1.** (A) The domain structure of STAT1 protein is shown. The residues affected by mutation are shown in red. The tyrosine phosphorylation site is present at amino acid 701. For the constitutively active form of STAT1, cysteine residues were introduced into Ala-656 and Asn-658. CC, coiled coil domain; DBD, DNA binding domain; LD, linker domain; SH2, Src homology 2 domain; TAD, transcriptional activation domain. (B) Total cellular proteins were prepared from the control, STAT1wt-, and STAT1ca-overexpressing NIH3T3 cells, and then subjected to western blotting using antibodies to total STAT1 and  $\beta$ -actin. (C) Total RNA from the control, STAT1wt-, and STAT1ca-overexpressing NIH3T3 cells were isolated, and *IRF1* mRNA levels were measured by qRT-PCR.



**Figure VI-8. Effects of STAT1ca expression on the activity of the *Neurog2* promoter.** (A) The Neurog2pro-Luc or the promoter-less (Mock) plasmid was transfected into the control or STAT1ca-overexpressing NIH3T3 cells. After 1 day, the cell lysates were subjected to a luciferase reporter assay. The relative luciferase activity is shown. (B) The Neurog2pro-Luc or Mock plasmid was transfected into the control or IFN- $\gamma$ -overexpressing NIH3T3 cells. After 1 day, the cell lysates were subjected to a luciferase reporter assay. The relative luciferase activity is shown. \*\*\* $P < 0.001$ ; n.s., not significant.

				Relative Luciferase Activity	Fold change	Statistical significance
[#1]	-540/+586	 Full length (1125bp)	C	1	-1.9	***
			STAT1ca	0.52±0.09		
[#2]	-468/+586	 (1055bp)	C	0.94±0.15	-2.4	***
			STAT1ca	0.40±0.08		
[#3]	-412/+586	 (999bp)	C	0.84±0.11	-2.2	***
			STAT1ca	0.38±0.03		
[#4]	-200/+586	 (787bp)	C	0.95±0.16	-2.4	***
			STAT1ca	0.40±0.08		
[#5]	-49/+586	 (636bp)	C	0.98±0.17	-2.6	***
			STAT1ca	0.37±0.05		
[#6]	-540/+314	 (855bp)	C	1.17±0.16	-2.2	***
			STAT1ca	0.53±0.08		
[#7]	-540/+129	 (670bp)	C	1.24±0.17	-2.3	***
			STAT1ca	0.54±0.11		
[#8]	Splicing site mutant	 (1125bp)	C	0.81±0.15	-2.1	***
			STAT1ca	0.38±0.10		

**Figure VI-9. Effects of STAT1 overexpression on different forms of the *Neurog2* promoters.**

Schematic diagrams are shown for eight different forms of the *Neurog2* promoter. The arrow indicates the transcription start site. The control or STAT1ca-overexpressing NIH3T3 cells were transfected with the respective reporter plasmids. After 1 day, the cell lysates were subjected to a luciferase reporter assay. The relative luciferase activity, fold change, and statistical significance are shown.

\*\*\*P<0.001.

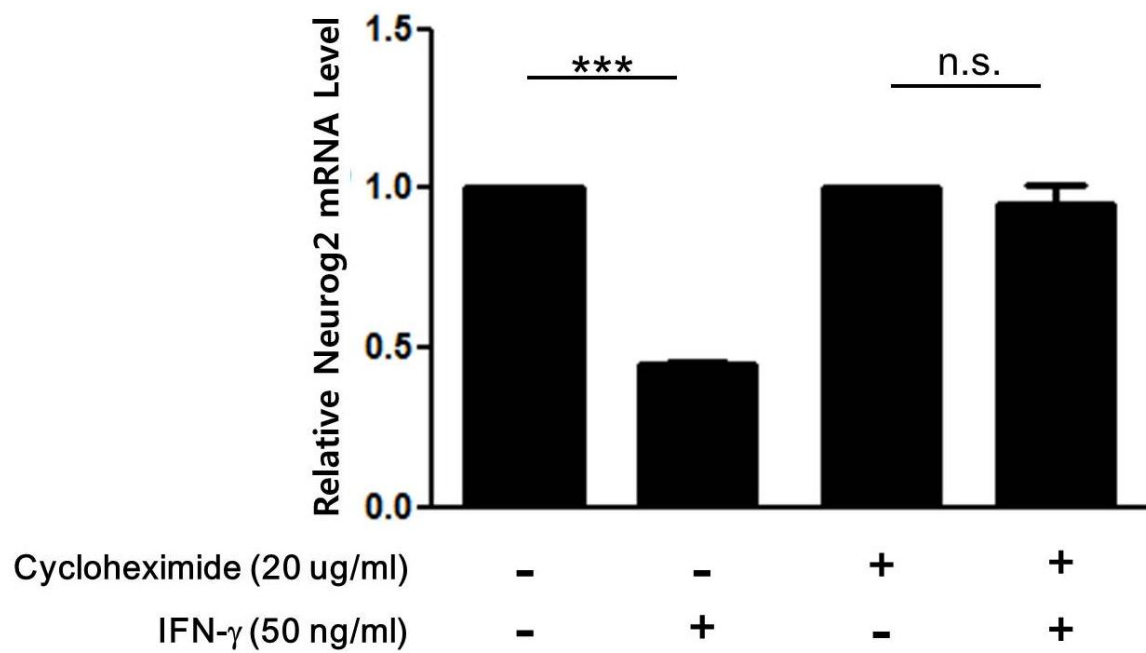
plasmid and a luciferase assay was performed after 1 day. There were no statistically significant differences in the basal activities of all the promoters tested (Fig. VI-9). Interestingly, the inhibitory effect of STAT1ca on the *Neurog2* promoter activity was observed in all eight forms of the promoter, with a 48–62% reduction. These results suggest that there might not be a cis-acting sequence interacting with STAT1 within the -540 to +586 bp region of the *Neurog2* genome, and that the reduction might be achieved via complex mechanisms, and not by simple promoter binding.

## **2.6 IFN- $\gamma$ -mediated inhibition of *Neurog2* expression requires *de novo* protein synthesis**

Many proteins are expressed upon STAT1 activation, and therefore, it is possible that STAT1 induces the expression of other transcription factors, which might in turn regulate the expression of *Neurog2*. To test whether the synthesis of other proteins is required for the regulation of *Neurog2* expression, primary NPCs were treated with a translational inhibitor, cycloheximide, and differentiated with or without IFN- $\gamma$ . IFN- $\gamma$  treatment resulted in the reduction of *Neurog2* expression by 55%, while this inhibitory effect was completely reversed upon cycloheximide treatment (Fig. VI-10). This data indicates that the IFN- $\gamma$ -mediated suppression of *Neurog2* expression may require *de novo* protein synthesis.

## **3. Discussion**

As described in the previous chapter, IFN- $\gamma$  treatment downregulates the expression of *Neurog2* that plays a crucial role in neuronal differentiation. To understand the precise relationship between IFN- $\gamma$  and neurogenesis, the molecular mechanisms underlying the IFN- $\gamma$ -mediated downregulation of *Neurog2* expression were investigated. The *Neurog2* promoter was cloned and its



**Figure VI-10. *De novo* protein synthesis is required for the IFN- $\gamma$ -mediated downregulation of *Neurog2*.** NPCs were differentiated in the presence of 20  $\mu$ g/mL cycloheximide and 50 ng/mL IFN- $\gamma$  for 12 hr. Total RNA was isolated and the *Neurog2* mRNA level was measured using qRT-PCR. \*\*P < 0.01; \*\*\*P < 0.001; n.s., not significant.



activity was measured in various experimental conditions by activating the STAT1 signaling pathway using different methods. In both the IFN- $\gamma$  treated and STAT1ca-expressing cells, the activity of the *Neurog2* promoter was nearly half of that in the control cells. To discern any possible artifacts that might result from the transient transfection assay where the plasmid is present in its extrachromosomal form, the reporter plasmid was integrated into the cellular chromosome. The activity of the integrated promoter was reduced to a similar level upon IFN- $\gamma$  treatment, indicating that there is approximately two-fold contribution of IFN- $\gamma$  to the *Neurog2* promoter activity.

To identify the STAT1 regulatory element in the *Neurog2* promoter, deletion mutations were introduced into the *Neurog2* promoter, and the effects of IFN- $\gamma$  on their activity were tested. Interestingly, the IFN- $\gamma$ -mediated inhibition of the *Neurog2* promoter activity was not rescued in any of the deletion forms. Moreover, there was an approximately five-fold difference in the magnitude of reduction in the level of the *Neurog2* mRNA expression and the promoter activity. Several other studies have also presented the similar case that showed up to ten-fold magnitude differences between the level of mRNA expression and the promoter activity [152-154]. These results could be due to differences between the experimental system and the natural condition as the transfection of a reporter plasmid might not ensure that the promoter is regulated in a natural way.

It is also highly likely that regions outside the *Neurog2* promoter are affected by IFN- $\gamma$ . In the spinal cord, it has been reported that the regulation of *Neurog2* can be achieved via cis-regulatory regions located thousands of base pairs upstream or downstream from the transcription start site [155]. These enhancers are highly conserved in different species, suggesting its role in gene regulation. The upstream regulator of *Neurog2*, Pax6, was found to interact with one of the enhancers, E1, which is located more than 7 kbp upstream from the transcription start site [145]. Retinoic acid response elements and a Gli binding site were also found in the E3 enhancer element, which is located more than 3 kbp downstream from the transcription start site [155, 156]. Another possible mechanism may be via epigenetic control. There have been yet no studies of the effects of IFN- $\gamma$  on this type of regulation, but IFN- $\gamma$  may contribute to changes in methylation or acetylation patterns of *Neurog2* as

the gene expression pattern in progenitor cells is often regulated by epigenetic factors [16, 17]. Changes in mRNA stability could also have led to the reduction in *Neurog2* expression, although there have been yet no studies reported. In addition, several other factors might play a role in the downregulation of *Neurog2* expression upon IFN- $\gamma$  treatment according to the data from cycloheximide experiment. As IFN- $\gamma$  treatment did not change the expression pattern of the factors those were reported to regulate *Neurog2* expression such as Pax3, Pax6, Hes1,  $\beta$ -catenin, and RP58 in primary NPCs (data not shown), other novel factors are believed to play a role. Overall, the data from this chapter suggested that the IFN- $\gamma$ -mediated reduction of *Neurog2* expression might involve complex mechanisms rather than an interaction between the promoter and one particular DNA-binding protein. Further investigations are required to determine the exact mechanism by which IFN- $\gamma$  abrogate the expression of *Neurog2* in primary NPCs.

## **CHAPTER VII**

### **Conclusion**

The primary aim of this thesis research was to determine the roles of stress-related factors in the initiation of neurogenesis. As the first step, I screened five genes, whose expression levels were previously known to be regulated under stressed condition. Their effects on the neuronal differentiation of NPCs were investigated using primary NPCs. The two factors with strong negative effects on neurogenesis, GSK3 $\beta$  and IFN- $\gamma$ , were selected and further characterized *in vitro* as well as *in vivo* using the UIGD technique.

GSK3, which has been reported to be upregulated in the case of prenatal alcohol exposure, is considered to be the master regulator of neurogenesis. However, the exact roles of the two isoforms of GSK3 have not been made clear. In this study, the functions of both isoforms were studied in primary NPCs and in the embryonic brain using various techniques such as chemical inhibitor, overexpression, and shRNAs. Through these experiments, it was revealed that only GSK3 $\beta$ , but not GSK3 $\alpha$ , inhibits embryonic neurogenesis. Furthermore, by treating rapamycin to GSK3 $\alpha$ - or GSK3 $\beta$ -knock-down cells, it was suggested that mTORC1 might differentially regulate the activity of GSK3 $\alpha$  and GSK3 $\beta$ . This is the first study to clarify the different roles of GSK3 $\alpha$  and GSK3 $\beta$  in the context of embryonic neurogenesis, as well as the relationship between mTORC1 and GSK3 in primary NPCs. These findings are believed to be useful for understanding the molecular function of GSK3 in general, as well as facilitating the development of new and effective therapeutic targets for the many diseases in which GSK3 is known to play a role.

Another detailed biological investigation was carried out on IFN- $\gamma$  which has been reported as the major cytokine released in the embryonic brain upon prenatal infection. Prior to this study, the role of IFN- $\gamma$  in embryonic neurogenesis and its downstream mechanism have been elusive. In this research, the function of IFN- $\gamma$  in the embryonic brain was tested for the first time, and the obtained results demonstrated that IFN- $\gamma$  has a negative role in neuronal differentiation *in vivo*. Moreover, it was found that IFN- $\gamma$  inhibits the neuronal differentiation of primary NPCs by reducing the NPC-neuronal transition via the JAK/STAT1 pathway, and repressing one of the proneural genes, *Neurog2*. To the best of my knowledge, this is the first study to suggest a role of IFN- $\gamma$  in the regulation of

proneural gene expression in differentiating NPCs. These findings may provide an interesting starting point for understanding the role(s) of IFN- $\gamma$  during brain development, and ultimately, in the neurodevelopmental disorders caused by congenital infections.

Many case reports have suggested that stress during pregnancy can significantly affect brain development in children. Gestational exposure to alcohol has been reported to affect multiple stages of brain development including neuronal differentiation, proliferation, migration and gliogenesis, leading to many neurological defects in newborn children, such as microcephaly, mental retardation, lack of focus and delayed development, which are collectively known as fetal alcohol spectrum disorders (FASDs) [157]. In the case of neurogenesis, alcohol has been known to inhibit it, reducing the number of cortical neurons; however, clear studies of the mechanism are lacking [158-162]. When a pregnant dam is exposed to alcohol, the alcohol crosses the placenta and directly affects gene expression in the fetus. Interestingly, several studies have reported that the PI3K/AKT/mTOR pathway is inhibited upon exposure to ethanol in mouse myocytes, rat hearts and human neuronal cells [163-165], while the activity of GSK3 has been reported to be increased in ethanol-treated rat cortical neuroblasts, and the hippocampus of adolescent (postnatal day 23) mice prenatally exposed to alcohol [166, 167]. According to these studies and the results from my thesis, one of the possible pathogenesises of FASDs would be due to the inhibition of the mTORC1 signaling pathway upon ethanol exposure, which would in turn activate GSK3 $\beta$ , leading to strong inhibition of embryonic neurogenesis. Although further *in vivo* studies are required, the findings from this thesis may provide a stepping stone to future mechanistic studies on FASDs.

Congenital infection is another prevalent cause that is thought to be responsible for brain developmental defects. Lymphocytic choriomeningitis virus, influenza virus, rubella virus, human cytomegalo virus, *Campylobacter rectus* and *Toxoplasma gondii* are some examples of the pathogens that are reported to cause severe neurological defects in infants who have survived congenital infection [57-62]. However, the exact mechanism of pathogenesis due to congenital infection has not yet been clearly defined. Several studies have suggested that the inflammatory response is the cause of neurological disorders [69], and IFN- $\gamma$  has been known to be increased upon infection in the fetal

brain [109, 111, 138]. Moreover, the elevated concentrations of IFN- $\gamma$  during the second trimester of pregnancy are significantly associated with an increased risk of a child being diagnosed with autism [168]. Interestingly, the level of plasma IFN- $\gamma$  has been found to be induced in autistic patients, suggesting a relationship between IFN- $\gamma$  and neurological disorders [169]. According to the results obtained in the present study, the abnormal expression of IFN- $\gamma$  in embryonic NPCs inhibits the expression of a major proneural gene, *Neurog2*. The downregulation of *Neurog2* not only inhibits the neuronal differentiation of NPCs, but may also lead to weak neuronal circuits as well as imbalances in the glutamatergic neurons and GABAergic neurons, which are the two main causes of neurodevelopmental diseases [139-141]. The results reported in this thesis may provide insights into the pathogenesis of fetal brain dysfunction after infection.

In this thesis, I investigated the roles of genes that are known to be upregulated during stressed conditions in embryonic neurogenesis, which occurs at the earliest stage of brain development. The results of this study clearly suggest GSK3 $\beta$  and IFN- $\gamma$  as strong negative controllers of embryonic neurogenesis. As the extent of neurogenesis during the early period of development is critical for shaping brain functions, the findings from this study may contribute to the understanding of basic brain development as well as the pathogenesis of many congenital neurological disorders. Although the experimental design employed in this research was efficient and yielded insightful data, further experiments are required to provide more relevant answers in the context of congenital brain defects. It would be valuable to determine whether the key behavioral defects those seen in FASDs, schizophrenia, or autism models (such as cognitive dysfunctions, increased anxiety level, or deficits in social interaction) appear when the activity of GSK3 $\beta$  or IFN- $\gamma$  is induced in the fetal brain, and whether these abnormal behaviors are rescued when the activity of GSK3 $\beta$  or IFN- $\gamma$  is blocked. Such results would provide powerful clues that can be applied in the development of a preventative drug or a treatment for brain disorders due to developmental defects.

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## 국문초록

태아가 스트레스에 노출될 경우 태어난 후에 심각한 신경 질환으로 이어질 수 있는 것이 밝혀져 있다. 많은 인자가 스트레스 상황과 관련이 있는 것으로 알려졌지만, 뇌 발달에서 그 역할이 명확하게 조사된 바는 없다. 이 연구는 실생활에서 흔히 일어나는 태아기 스트레스 중, 알코올 섭취와 바이러스 감염에 의해 태아 뇌에서 활성화된다고 알려진 다섯 개 유전자 (Nrf2, GSK3 $\alpha$ , GSK3 $\beta$ , IFN- $\gamma$ 와 SC4MOL)를 선정하고, primary embryonic day (E) 14.5 생쥐 신경 전구세포 (neural progenitor cells: 이하 NPCs)에서 그 영향을 조사하는 것으로 시작되었다. 레트로바이러스를 사용하여 각각의 유전자를 NPCs에서 과발현시킨 후, TuJ1 염색을 통해 분화된 신경세포의 비율을 분석하였다. GSK3 $\beta$ 와 IFN- $\gamma$ 는 과발현되었을 경우 TuJ1-양성 (TuJ1<sup>+</sup>) 세포가 크게 감소하는 것을 관찰하였다. 이는 이 두 유전자가 신경세포의 분화를 강하게 억제한다는 것을 의미하는 것으로서, 이후 다양한 기법들을 이용하여 신경세포 분화에서 이 두 인자의 역할과 기전을 조사하였다.

GSK3는 NPCs의 증식 및 분화를 조절하는 중요한 역할을 하는 것으로 잘 알려져 왔으나 두 개 동형단백질(isoform)인 GSK3 $\alpha$ 와 GSK3 $\beta$  각각의 역할에 대해서는 논란이 있었다. 따라서 이 연구에서는 신경 분화의 과정에서 GSK3 $\alpha$  및 GSK3 $\beta$ 의 기능에 차이가 있는지를 조사하는 데 초점을 두었다. GSK3 억제제 (SB216763) 처리 시, NPCs의 TuJ1<sup>+</sup> 신경세포의 증가가 관찰되었는데 이는 GSK3가 신경세포 분화를 억제한다는 것을 의미한다. GSK3 $\beta$ 의 knock-down은 TuJ1<sup>+</sup> 세포의 수를 증가시킨 반면, GSK3 $\alpha$ 의 knock-down은 아무런 영향을 주지 않았다. GSK3 $\beta$  (Y216F)를 과발현시켰을 경우에는 신경세포 분화의 억제 효과가 나타나지 않았는데 이는 GSK3 $\beta$ 의 kinase 활성 자체가 그 역할에 중요하다는 것을 나타낸다. 초음파 영상을 이용하여 E9.5 생쥐의 뇌에 GSK3 $\beta$  발현 레트로바이러스 벡터를 전달한 *in vivo*



실험에서도 이와 유사한 결과가 관찰되었다. 또한, SB216763 를 처리하면 rapamycin 처리에 의해 억제된 신경세포 분화가 다시 회복되는 것이 관찰되었다. 이러한 결과는 (GSK3  $\alpha$  가 아니라) GSK3 $\beta$ 만이 신경세포 분화를 억제하며, 이는 mTORC1 신호 전달 경로의 하위 단계에서 작용하는 것을 의미한다.

IFN- $\gamma$ 는 바이러스 감염 시에 분비되는 대표적인 사이토카인 중 하나이다. 숙주 면역 반응에서 IFN- $\gamma$ 가 중요하다는 것은 잘 알려졌다으나 뇌 발달 측면에서 수행된 많은 *in vitro* 연구들은 상반되는 결과를 보였고, *in vivo* 실험은 전혀 없었다. E14.5 의 NPCs 에 IFN- $\gamma$ 를 처리하였을 경우, TuJ1<sup>+</sup> 신경 세포의 비율이 현저히 감소하였고, Nestin<sup>+</sup> NPCs 의 비율이 크게 증가하였다. 초음파 영상을 이용하여 E9.5 생쥐의 뇌에 IFN- $\gamma$  발현 레트로바이러스 벡터를 전달한 *in vivo* 실험에서도 유사한 결과가 나타났다. JAK 억제제인 Ruxolitinib 을 처리하거나 STAT1 의 발현을 knock-down 시켰을 경우에는 IFN- $\gamma$ 로 인한 신경세포 분화의 억제가 사라지는 것이 관찰되었다. 흥미롭게도 IFN- $\gamma$  처리를 한 NPCs 에서는 전신경 유전자 (proneural gene)의 하나인 *Neurogenin2* (*Neurog2*)의 발현이 억제되었으며, *Neurog2*를 과발현하는 세포에서는 IFN- $\gamma$ 의 효과가 나타나지 않았다. IFN- $\gamma$ 처리와 항상 활성을 가지도록 조작된 STAT1 돌연변이 단백질의 과발현은 *Neurog2* 프로모터의 활성을 약 절반으로 감소시켰다. 이 결과는 IFN- $\gamma$ 가 JAK/STAT1 신호전달 체계를 통해 *Neurog2*의 발현을 프로모터 수준에서 부분적으로 조절함으로써 NPCs 의 신경세포 분화를 억제한다는 사실을 의미한다.

이 연구를 통해 GSK3 $\beta$ 는 mTORC1 신호체계의 하위단계에서, IFN- $\gamma$ 는 JAK/STAT1 신호체계의 상위단계에 작용하여 신경세포 분화를 강하게 억제한다는 사실을 밝혔다. 이를 통해 신경세포 분화관점에서 GSK3 두 개 동형단백질의 역할을 최초로 명확히 밝혔으며, IFN- $\gamma$ 의 효과를 *in vivo* 에서 처음으로 확인하고, 하위 기전을

제시하였다. 이 논문의 연구 결과는 여러 스트레스 관련 인자들이 초기 뇌 발생에 미치는 영향과 작용 메커니즘을 이해하는데 기여할 수 있을 것으로 생각된다.

**핵심어:** 신경발생, 신경줄기세포 분화, 태아기 스트레스, Nrf2, GSK3, IFN- $\gamma$ , SC4MOL